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Mecynorhina ugandensis (Moser, 1907) - Uganda

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Taxonomy and biology of the African Goliathini Krikken, 1984 (Coleoptera Cetoniidae). The family of Cetoniidae Leach, 1815 includes species of beetles particularly diversified in the morphology, often with large dimensions, and with an important and little known pre-imaginal biology and numerous taxonomic aspects to be verified. In particular, the Goliathini are one of the 12 tribes in which the subfamily of the Cetoniinae is divided. *Goliathus* Lamarck, 1801 (named after the biblical giant Goliath), which is about 11 cm long, is one of the largest species of beetles in the world. The Goliathini are characterized by an obvious sexual dimorphism. The males have, almost always, clypeal horns, often bifurcated. They are widespread in the tropical areas of the African continent (78 genera), while only 2 genera with three species in the American continent, Mexico, follow a paleoamerican dispersion model. The adults of Goliathini are flyers and feed on sugary substances; the larvae are saproxylic. They are entirely dependent for its larval growth and development on large, dead trees, often still standing and in an advanced stage of decomposition. By degrading dead wood, they are of great importance in the ecology of forests. Unfortunately, the African forests are currently under unprecedented threats, and many Goliathini are included in numerous lists of endangered and protected species. The genus *Mecynorhina* Hope, 1837, (photos) listed about 10 species subdivided into 4 subgenres, spread throughout the tropical African region.

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Identification of subspecies and parentage relationship by means of DNA fingerprinting in two exemplary of *Pan troglodytes* (Blumenbach, 1775) (Mammalia Hominidae)

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ABSTRACT Four chimpanzee subspecies (Mammalia Hominidae) are commonly recognised: the Western Chimpanzee, *P. troglodytes verus* (Schwarz, 1934), the Nigeria-Cameroon Chimpanzee, *P. troglodytes ellioti,* the Central Chimpanzee, *P. troglodytes troglodytes* (Blumenbach, 1799), and the Eastern Chimpanzee, *P. troglodytes schweinfurthii* (Giglioli, 1872). Recent studies on mitochondrial DNA show the incorporation of *P. troglodytes schweinfurthii* in *P. troglodytes troglodytes*, suggesting the existence of only two subspecies: *P. troglodytes troglodytes* in Central and Eastern Africa and *P. troglodytes verus*-*P. troglodytes ellioti* in West Africa. The aim of the present study is twofold: first, to identify the correct subspecies of two chimpanzee samples collected in a Biopark structure in Carini (Sicily, Italy), and second, to verify whether there was a kinship relationship between the two samples through techniques such as DNA barcoding and microsatellite analysis. DNA was extracted from apes' buccal swabs, the cytochrome oxidase subunit 1 (COI) gene was amplified using universal primers, then purified and injected into capillary electrophoresis Genetic Analyzer ABI 3130 for sequencing. The sequence was searched on the NCBI Blast database. In addiction, the microsatellite analysis was performed on the same machine for parentage detection among samples, and data were analyzed with GenMapper software. Our results show that both samples were *P. troglodytes troglodytes*, while the analysis of the microsatellite results in an unclear relationship between two chimpanzee samples.

KEY WORDS Hominidae; *Pan*; Africa; DNA; cytochrome oxidase; evolution.

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INTRODUCTION

Cellular Biology and Molecular Genetics have assumed over time a greater role in species identification. The identification was based on the assumption that there are no individuals (except homozygous twins) who have exactly the same genome. The sequence of mitochondrial cytochrome C oxidase subunit 1 (COI), often referred to as a "DNA barcode" (Hebert et al., 2003), contain approximately 648 base-pair in almost all the species and can serve as the standard barcode for almost all animals.

DNA barcode amplicons are typically obtained by PCR using standardized and universal primer sets; there are approximately five million COI barcode sequences in GenBank and/or BOLD (Barcode of Life) databases in about 280,000 species.

In addition to "DNA barcode", the study of genetic diversity among species or subspecies can be obtained analyzing the combination of a group of microsatellites loci with relative alleles frequencies extracted from genomic sequences.

The possibility of carrying out genetic traceability analysis on biological samples in the framework of surveillance programs, represents without doubt a strong deterrent for illegal commercial procedures, illegal hunting, and protected species commercialization. Conducting this type of investigation requires a technique that combines sensitivity and high discriminating power, so as to allow researchers to use it even on minimum sample quantities and to trace or identify an individual in a univocal way and with a low margin of error.

By means of microsatellites, we can detect parentage relationship among samples, but also carry out population studies and shed light on migration and evolutionary processes.

Knowing the genetic profile of a single animal in relation to certain polymorphisms allows to:

• ascertain the pedigree by genetic investigation of paternity and / or maternity;

• tackle suspected cases of poaching, acts of cruelty, and illegal imports of protected animals;

• identify the species and / or determine the sex, helping to safeguard biodiversity.

Knowledge of population relationships might also facilitate the use of the limited resources available for conservation efforts (Schonewald-Cox et al., 1983; Avise, 1996), and might help in guiding breeding programs of chimpanzees kept in captivity (see Witzenberger & Hochkirch, 2011; Hvilsom et al., 2013).

Genetic data on mitochondrial DNA (Gonder et al., 2006), analyses of complete genomes (Prado-Martinez et al., 2013), and on autosomal microsatellites (Fünfstück et al., 2015) suggest that the subspecies form two distinctive groups: one group includes *P. troglodytes verus* and *P. troglodytes ellioti* in West Africa and the other group includes *P. troglodytes troglodytes* and *P. troglodytes schweinfurthii* in Central and Eastern Africa.

Fischer et al. (2006) argue that, based on their work on nuclear DNA and considerations on morphological and behavioral similarity, the difference between chimpanzees is too small to justify the distinction in subspecies.

Later studies, including more mtDNA haplotypes, once more did not find consistent support for monophyly of *P. troglodytes troglodytes* and *P. troglodytes schweinfurthii* (Gagneux et al., 2001; Gonder et al., 2006). In addition, in the study by Gonder et al. (2006), no fixed nucleotide differences distinguishing the haplotypes of Central and Eastern chimpanzees were detected.

Other bibliographies consulted are: Boesch & Boesch, 1993; Sakura, 1994; Bard, 1995; Jones et al., 1996; Goldberg & Wrangham, 1997; Gagneux et al., 1999; Mitani et al., 2000; Butynski, 2003; Marsh, 2003; Poulsen & Clark, 2004; Prufer et al., 2012.

MATERIAL AND METHODS

Two samples of chimpanzee (*Pan troglodytes*) from the Bioparco di Sicilia, Carini (Sicily, Italy), both coming from a Belgian circus, were analyzed in April 2017.

Eight DNA samples were collected from buccal swabs, four samples for each chimpanzee. The two specimens have been identified as: first individual, named Mango (MN), born in 2000; second individual, named Whiskey (WY), born in 1998.

The samples, numbered and subdivided into different plastic bags, were deposited in a transport box and placed in a cooler bag, and the following day brought to the "Istituto Zooprofilattico Sperimentale della Sicilia", Palermo (Italy). DNA extraction was performed through the kit E.Z.N.A. Tissue DNA Kit Protocol - Whole Blood and Body Fluids. Quantification of DNA extracted was performed using NANODROP 1000 spectrophotometer from THERMO SCIENTIFIC. The COI gene was amplified by PCR using the AmpliTaq Gold™ DNA Polymerase Kit (Applied Biosystems) and the specific primers Cyt-1 (F) CCAATGATATGAAAAACATCGTT and Cyt-2 (R) GCCCCTCAGAATGATATTTGTCCTC for a final size of amplificate of 474 base pairs. The mixture was optimized as follows: 1X PCR Buffer 5 µl, 2 mM MgCl2 4 µl, 10 mM dNTP mixture 2 µl, 0.6 pmol/µl cyt B1 0.5 µl, 0.6 µl cyt B1 0.5 µl , 0,6 µl cyt B2 0.5 µl, 0.03 U/µl taq Polymerase 0.3 µl and water to 50 µl final volume. The amplification was

optimized in accord to the manufacturer (Thermo), in a 9700 thermal cycler (Applied Biosystems) with an initial denaturation step at 94 °C for 8 min, followed by 40 cycles, primer annealing at 53 °C for 50 s, and elongation at 72 °C for 1 minute and a final extension step at 72 °C for 7 minutes. All gene amplification reactions were visualized on 2% agarose gel (GellyPhor Euroclone), prepared by dissolving the 0.5X TBE agar and the DNA bands of interest displayed through an UV image acquisition system, ChemiDoc BioRad (Biotec 206). The samples of amplified DNA were subjected to purification through the "GFX PCR DNA and gel band purification kit". Once the purified ones were obtained, the sequence PCR was performed, with the "Bigdye Terminator Cycle Sequencing Kit" (Applied Biosystems), considering a reaction volume of 20 µl for each sample. The sequence products were then purified through the "Big Dye Xterminator Purification KIT" kit and injected into the ABI Prism 3130 DNA sequencer (Life technologies).

The sequences obtained were searched on the Basic Local Alignment Search Tool (BLAST) Database for species identification. Only sequences with a low e-value and high degree of identity were retained. Further analyzes for the identification of microsatellites were carried out. Initially, after the DNA extraction from buccal swabs, samples were adjusted for their concentration in ng/µl after dilution with the corresponding TE at 0.1%. The mix was constituted in according to Kit "AMPF1 STR Identifiler PCR Amplification". The amplification program includes an initial 95 °C incubation step for 11 minutes, a denaturation phase at 94 °C for 1 minute, an annealing step at 59 °C for 1 minute, an initial extension at 72 °C for 1 minute, an extension final at 60 °C for 60 minutes. Genetic profiles obtained from the microsatellites analysis were analyzed using the GeneMapper ID v4.0 software. Applied Biosystems multicolour fluorescent dye technology enables the analysis of multiple loci, including loci that have alleles with overlapping size ranges. The alleles for the superimposed loci are distinguished by the labeling of specific primers for locus with different colored dyes. Multi component analysis is the process that separates the different colors of fluorescent dye into distinct spectral components. The four dyes used in the Identifiler kit for labeling the samples are shown in Table 1.

RESULTS AND DISCUSSION

The amplification of the COI gene in eight isolates taken from two samples of *P. troglodytes* gave a specific band on agarose gel. The size of amplified fragment was 508 bp (Fig. 1).

NCBI BLAST database search of the sequence gave a similarity with species *Pan troglodytes troglodytes* for both Wishy and Mango samples with 99% of identity.

The microsatellites fragment were analyzed using the GeneMapper ID software. All electropherograms derived from the fragment analysis of samples of *Pan troglodytes* for each locus are reported in figures 2–7. We excluded the fluorescent dye PET® because it did not give any peak.

With the VIC® dye, in the two figures (Figs. 4, 5), the electropherograms of each locus, respectively of Whisky and Mango, were represented.

With the NED™ dye, in the two figures (Figs. 6, 7), the electropherograms of each locus, respectively of Whisky and Mango, were represented.

In Table 2, the alleles for each locus are reported for all samples. Sample 3 of Whisky didn't present

Dye	Locus
6-FAM	D8S1179 D21S11 D7S820 CSF1PO
VIC	D3S1358 TH01 D13S317 D16S539 D2S1338
NED	D19S433 vWA TPOX D18S51
PET	Amelogenina D5S818 FGA

Table 1. Loci list and relative dye employed.

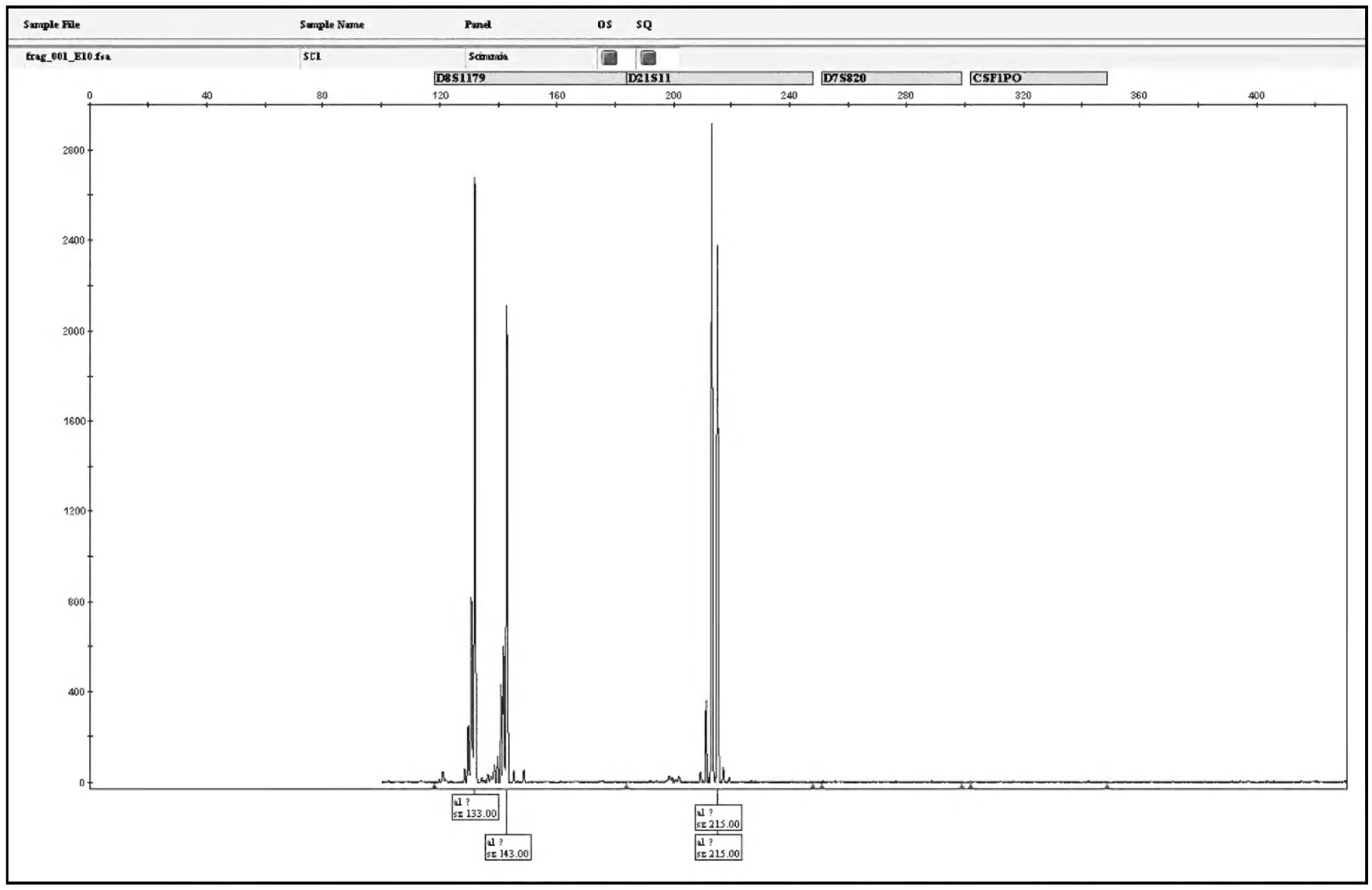

Figure 1. Electropherograms of the Whisky sample with 6- FAM ™ dye.

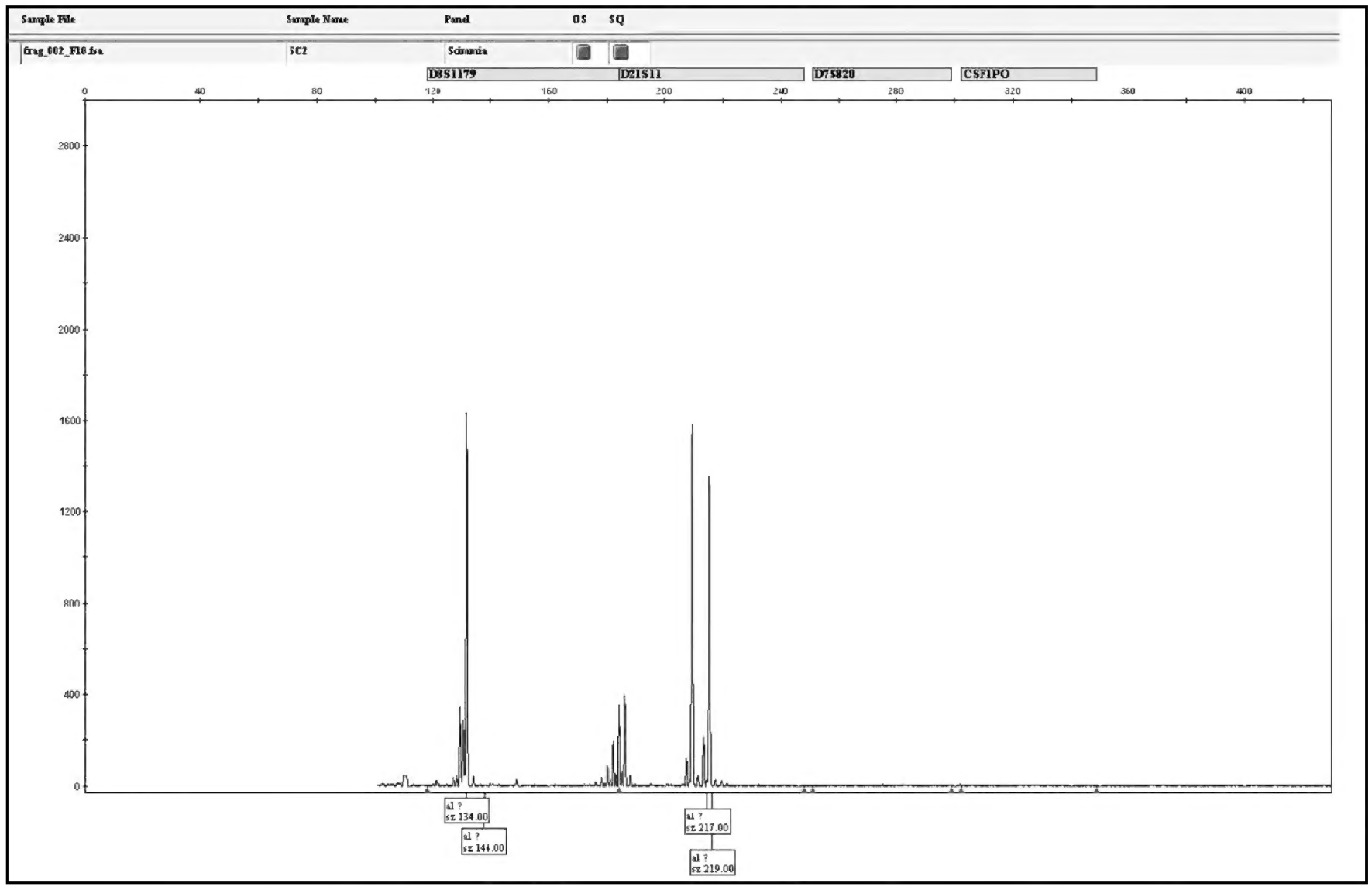

Figure 2. Electropherograms of the Mango sample with 6- FAM ™ dye.

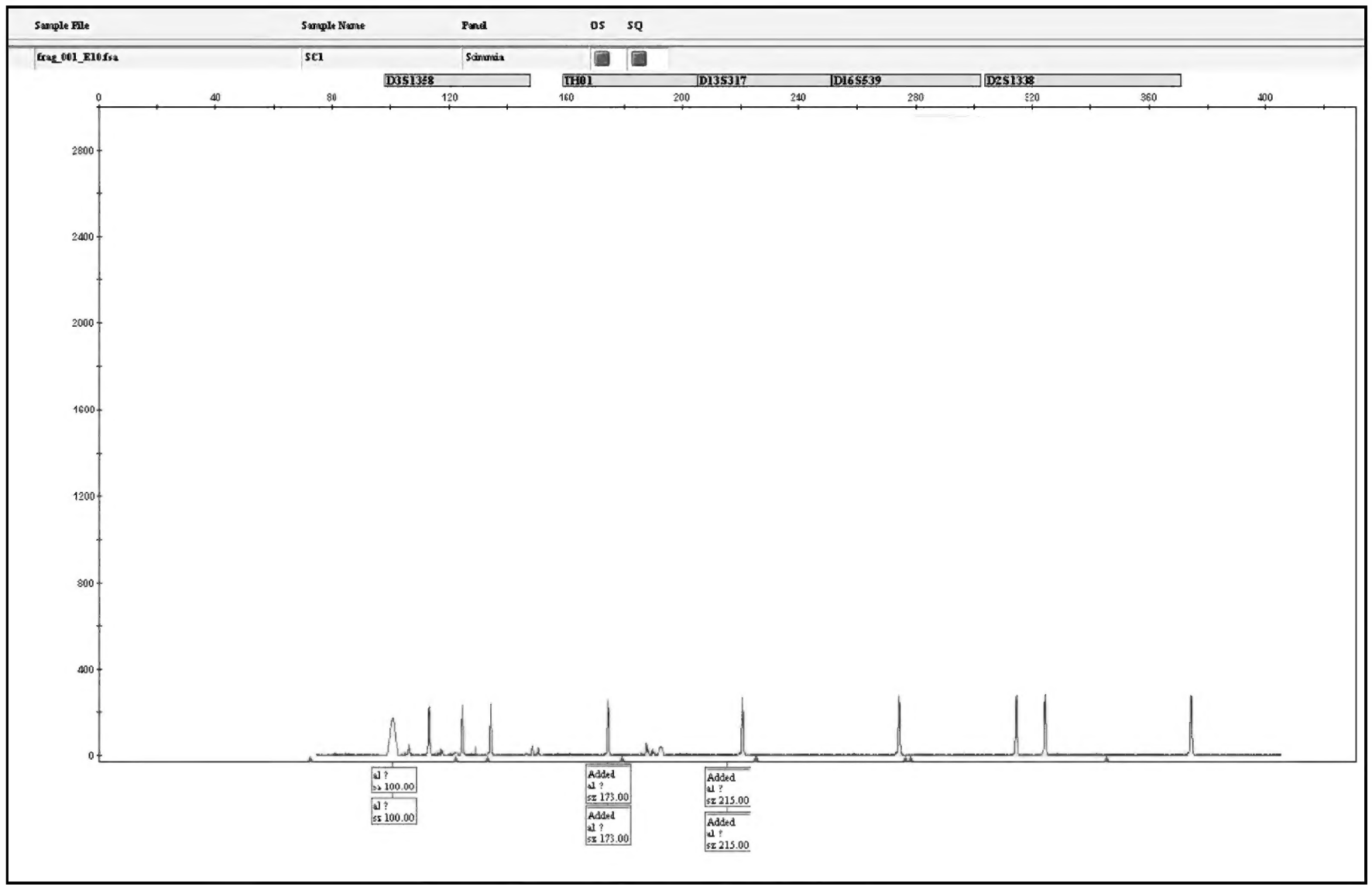

Figure 3. Electropherogram of the Whisky sample with VIC® dye.

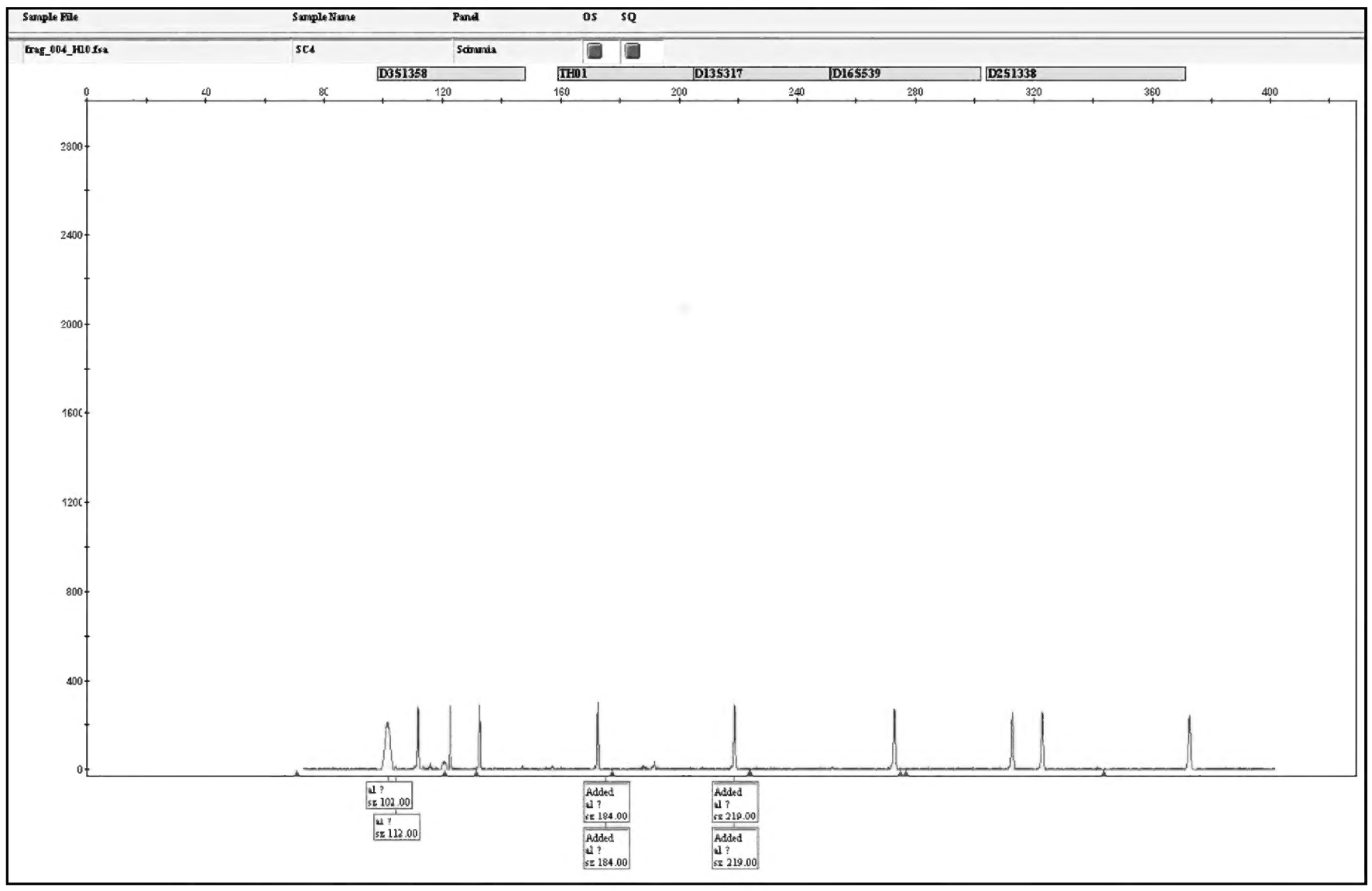

Figure 4. Electropherogram of Mango sample with VIC® dye.

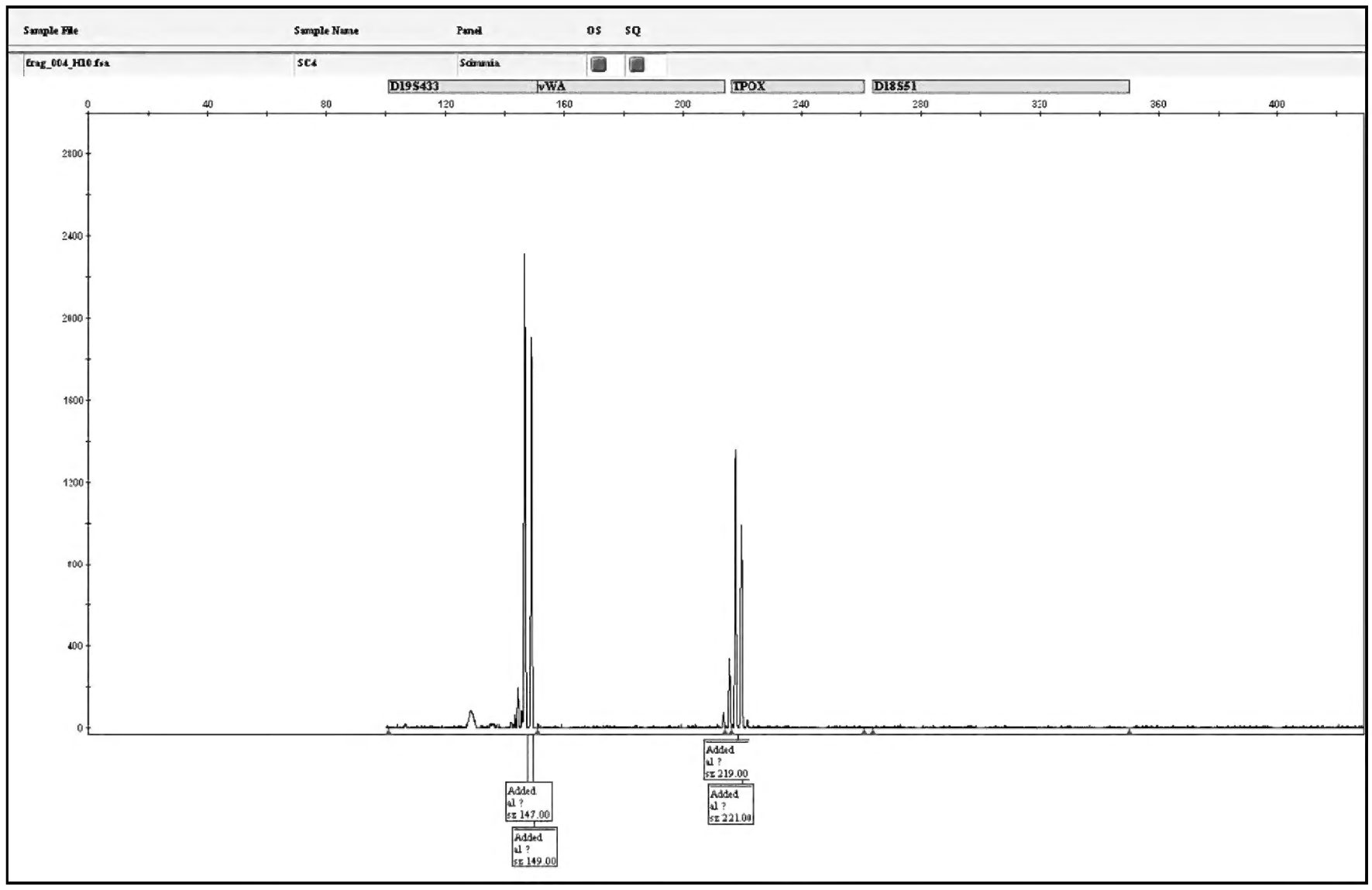

Figure 5. Electropherogram of Whisky samples with NED™ dye.

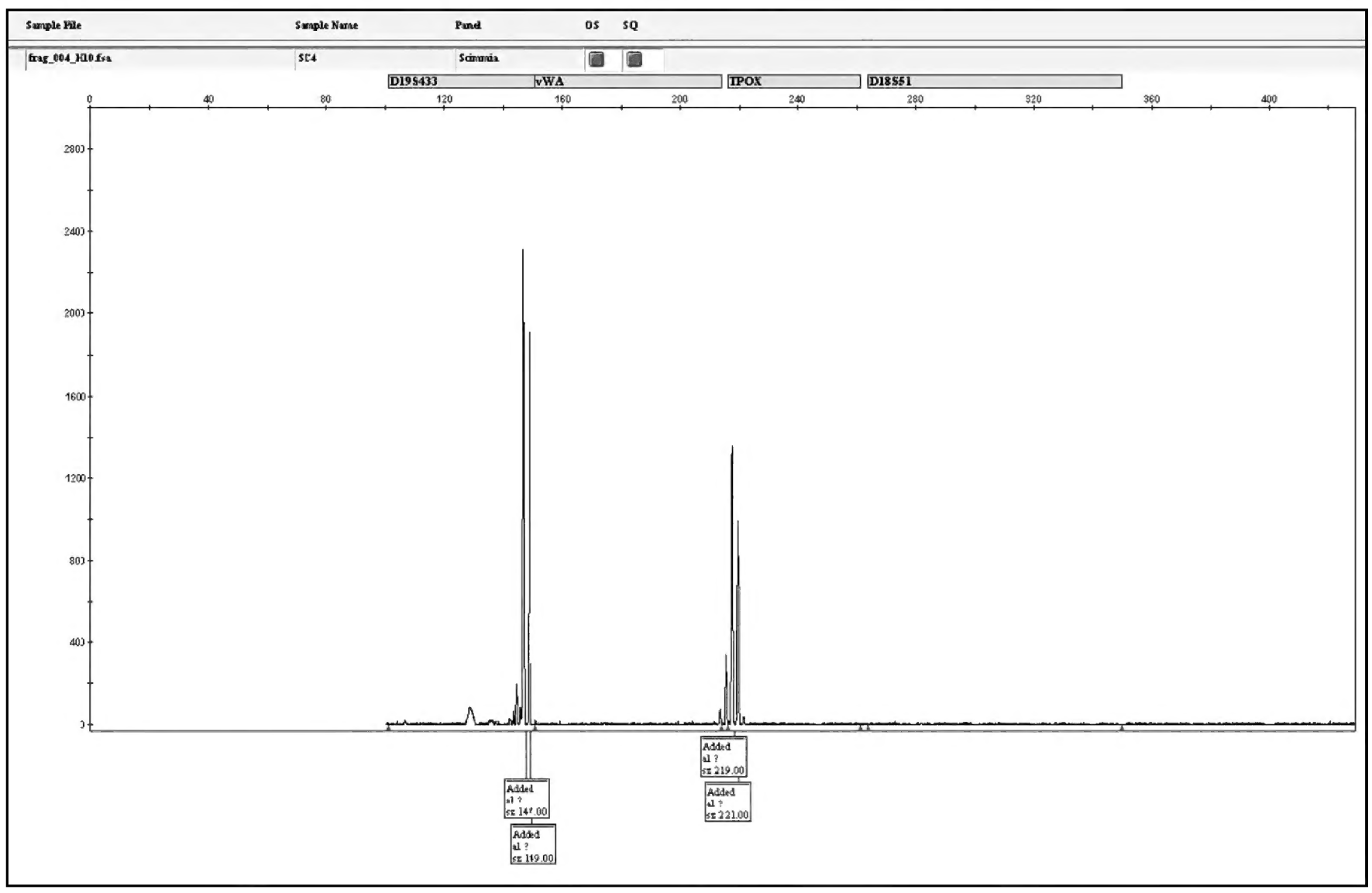

Figure 6. Electropherogram of Mango samples with NED™ dye.

LOCUS	DYE	DYE	1 WHISKY	3 WHISKY	5 WHISKY	7 WHISKY	2 MANGO	4 MANGO	6 MANGO	8 MANGO
D8S1179	Fam	Blue	133-143	N	133-143	133-143	134-144	134-144	134-144	134-144
D21S11	Fam	Blue	215-215	N	215-215	215-215	217-219	217-219	217-219	217-219
D7S820	Fam	Blue	N	N	N	N	N	N	N	N
CSF1PO	Fam	Blue	N	N	N	N	N	N	N	N
D3S1358	Vic	Green	100-100	N	100-100	100-100	102-112	102-112	102-112	102-112
TH01	Vic	Green	173-173	N	173-173	173-173	184-184	184-184	184-184	184-184
D13S317	Vic	Green	215-215	N	215-215	215-215	219-219	219-219	219-219	219-219
D16S539	Vic	Green	N	N	N	N	N	N	N	N
D2S1338	Vic	Green	N	N	N	N	N	N	N	N
D19S433	Ned	Yellow	147-149	N	147-149	147-149	147-149	147-149	147-149	147-149
vWA	Ned	Yellow	N	N	N	N	N	N	N	N
TPOX	Ned	Yellow	217-221	N	217-221	217-221	219-221	219-221	219-221	219-221
D18S51	Ned	Yellow	N	N	N	N	N	N	N	N

Table 2. List of alleles for each locus in the two examined chimpanzees.

any peak. For the remaining seven samples, the two Mango and Whisky groups of replicates presented the same size for each locus and so we grouped it in two samples. We noted also that six loci (D7S820, CSF1PO, D16S539, D2S1338, vWA, and D18S51) do not give any result for all the samples, probably due to non specificity of the human locus primer used for the *P. troglodytes* species.

We observed that locus D19S433 share the same pair of allele between the two replicates (147 and 149) and that locus TPOX share one allele between the two groups of samples (221).

Despite the fact that Mango and Whisky had some locus allele size in common and in consideration of the fact that six loci didn't give any results, we can not assert a parentage relationship among two chimpanzees only based on these data.

CONCLUSIONS

Pan troglodytes s.l. is the most abundant, protected, and widespread of the great apes, the declines that have occurred are expected to continue, satisfying the criteria for an Endangered listing (Oates, 2006). Due to high levels of poaching, infectious diseases, and loss of habitat and habitat quality caused by expanding human activities, this species is estimated to have experienced a significant population reduction in the past 20–30 years and it is suspected that this reduction will continue for the next 30–40 years. Furthermore, zoonosis and disease outbreaks present significant risks; there is, for example, evidence that *Ebolavirus* will continue to spread in some parts of the Chimpanzee's geographic range (Walsh et al., 2005).

Actually, it is considered an Endangered species (EN) (Humle et al., 2016).

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Molecular techniques employed to trace the Sicilian ovines

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ABSTRACT Genotyping strategies are aimed at defining the genetic profile of individuals through the identification of STRs sequences. The applied methodologies are able to ensure the traceability of the meat along the production chains and the control of the correct animal sampling on the farms. However, the discriminative capacity of alleles is studied through the establishment of the allelic frequency in the ovine population of the territory. This may depend on factors such as race, degree of inbreeding, and local selections. In the research of genetic identity in particular, it is exploited that the probability that two different individuals possess the same genetic pattern is equal to the frequency of that genotype in the population under examination and that the frequency of a genotype characterized by more loci is equal to the product of the frequencies of each single genotype (locus) observed. Therefore, we set the task of fixing and tabulating the data of the genetic profiles of the autochthonous breeds that can then be exploited for the traceability investigations of the animals, according to the application of specific algorithms. In practice, we aim to establish and create the starting point for the interpretation of all the genetic data obtained from the analysis of the Sicilian ovine population, whatever the application to do with it. The ultimate goal of this work is the elaboration of allelic panels typical of the sheep populations that represent the starting point for all genetic tests of forensic investigations. In fact, the discovery of particular alleles identify the tabulated frequency representing the genetic variability distribuited in the region. This has the effect of minimizing the identification errors that are spread in the animal population. We can state that from the analysis of allele frequencies developed by Genalex we can obtain expected heterozygosity data according to Hardy-Weinberg law and the obtained heterozygosity data typical for native breeds. All the allele frequencies were employed to create a database containing all the genotypes. These data were useful in the forensic field for the attribution of the kinships in the sheep.

KEY WORDS Molecular genetics; Sicilian ovine; DNA; cytochrome oxidase; evolution.

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INTRODUCTION

Molecular genetics in forensic science finds application not only in the human field, but also in the veterinary, where such applications have allowed the development of multiple tools to ensure the protection of public health in the veterinary sector.

In this regard, there is a growing interest in the application, in the field of animal health, of the skills and methodologies developed in the field of individual genetic traceability for the determination

of the genetic profile of an individual through the use of DNA polymorphisms.

Knowing the genetic profile of a single animal in relation to a certain polymorphism allows to:

• ensure complete traceability of the meat along the entire supply chain, from primary production to sales to consumers, with particular reference to products that have specific food qualities;

• ascertain the "pedigree" of heads of interest through genetic investigation of paternity and/or maternity;

• ascertain the identity of an animal found as a result of loss or in cases of abigeats; tackle suspected cases of poaching, acts of cruelty, and illegal imports of protected animals;

• identify the species and/or determine the sex;

• identify if an animal died in the barn or on the street;

• resolve cases of judicial or veterinary police.

Furthermore, the availability of effective, rapid, and cheap genetic identification systems for every sheep can be extremely useful in order to implement epidemiological control programs and surveillance of infectious diseases. The possibility to carry out genetic traceability analysis on biological samples collected for serological controls, in the context of surveillance programs, is undoubtedly a strong deterrent for the incorrect sampling procedures. This type of investigation requires a technique that combines sensitivity and high discriminating power, so as to allow researchers to use it even on minimum sample quantities and to trace and / or identify an individual in a univocal way and with a low margin of error.

MATERIAL AND METHODS

The genetic analysis will be carried out on samples of peripheral blood with EDTA anticoagulant, taken from the sheep, and sent to the reference laboratories for the territories under the jurisdiction of the Experimental Zooprophylactic Institute of Palermo. Arbitrarily, 480 samples were considered as representative of the breed variety of the Sicilian territory for the control of genetic susceptibility to scrapie with molecular analysis. The data that are generated, for their interpretation, require a mathematical matrix that takes into account the allelic distribution throughout the population, so that each allele found, is then identified with a frequency index. Genomic DNA extracted from blood with the Genelute Blood Genomic DNA Kit from Sigma allows the identification of every animal and therefore can be useful in document traceability in animal products of sheep origin. A PCR was used for the amplification process, on which an end point study was performed to assess the presence of the sequences of interest, according to an AmpliTaq Gold™ 360 DNA Polymerase Kit which provides a genetic system based on the simultaneous amplification of the STR markers of interest by PCR, and also allows sequence analysis for capillary electrophoresis. The Kit contains:

Primer mix,
PCR Buffer,
dNTP mix,
DNA control,
Taq Gold DNA Polymerase.

The Mix primer is the mixture of the primers forward and reverse, for each locus to be analyzed. For each pair one of them is marked with a fluorescent dye of the type: FAM, HEX, ROX. Specific primers for different loci that fall in overlapping dimensional ranges are however diversifiable thanks to different fluorochrome markings. For amplification, 11 STR dinucleotide markers were recommended, for identification purposes in the sheep, by ISAG (International Society of Animal Genetics) and listed below in Table 1 together with the fluorescent molecule with which they are labeled after PCR, the resulting color, the number of alleles found in the Sicilian population, and the interval of length of alleles for each locus.

The ISAG markers, validated at European level for their information, are used by independent laboratories all over Europe as a basic panel of standard markers for the analysis of sheep genotypes. Within each range, for a single locus, it is possible to find one or two peaks between those coded in Table 2 that collects all the allelic groups. For each sample analyzed, 2 µl of DNA at the concentration of 20 ng/ml, prepared in the previous phases, was amplified in a final volume of 25 µl of the reaction mixture formed by 2 mixes. The mix 1 and 2 were composed as follow: 12.5 µl of Master mix, 2.5 µl primer mix, 10 µl water. The amplification program provides 95 °C for 5 minutes, 95 °C for 30 seconds,

63 °C 90 seconds, 72 °C 30 seconds, 60 °C 30 minutes, 4 °C kept in a final volume of 30 µl. The same amplification program is used for mix 2, with the only difference being the annealing temperature at 56 °C for 90 second. The amplifications were conducted on the thermal cycler "GeneAmp PCR system 9700 (Applied-Biosystems)". After the PCR, the product should be diluted 1:10 (25 µl of amplified + 225 µl of deionized water). A volume of 2 µl of PCR products were added to the formammide and the internal size standard (Rox). This sample mixtures were loaded in the sequencer in opportune order: (see Nei et al. (1983), Guo & Thompson (1992), MacHugh et al. (1998), Blott et al. (1999), Cornuet et al. (1999), Roques et al. (1999), Ciampolini et al. (2000), Pritchard et al. (2000), Bjørnstad & Røed (2001), Hansen et al. (2001), Caballero & Toro (2002), Maudet et al. (2002), Dieringer & Schlotterer (2002), Korstanje et al. (2003), Mburu et al. (2003), Queney et al. (2004), Rosenberg (2004), Chantry-Darmon et al. (2005), Excoffier et al. (2005), Gutirrez et al. (2005), Barcaccia & Falcinelli (2006), Whitlock et al. (2008), ISAG/FAO Standing Committee (2009).

PROG	LOCI	DYE	SIZE	SEQUENCE FORWARD (Fwd)	SEQUENCE REVERSE (Rev)
1	OV OarFCB011-FORW	FAM	120-150	GCAAGCAGGTTCTTTACCACTAGCACC	GGCCTGAACTCACAAGTTGATATATCTATCAC
2	INRA0063	FAM	160-210	GACCACAAAGGGATTTGCACAAGC	AAACCACAGAAATGCTTGGAAG
3	HSC	FAM	260-300	CTGCCAATGCAGAGACACAAGA	GTCTGTCTCCTGTCT TGTCATC
4	OarCP0049	HEX	80-140	CAGACACGGCTTAGCAACTAAACGC	GTGGGGATGAATATTCCTTCATAAGG
5	OarFCB0304	HEX	150-190	CCCTAGGAGCTTTCAATAAAGAATCGG	CGCTGCTGTCAACTGGGTCAGGG
6	CSRD0247	HEX	210-260	GGACTTGCCAGAACTCTGCAAT	CACTGTGGTTTGTATTAGTCAGG

Table 1. Sequences of ISAG loci and primers for the MIX1 (panel locus multiplex1).

PROG	LOCI	DYE	SIZE	SEQUENCES FORWARD (Fwd)	SEQUENCES REVERSE (Rev)
1	OarFCB0020	FAM	90-120	GGAAAACCCCCATATATACCTATAC	AAATGTGTTTAAGATTCCATACATGTG
2	D5S2	FAM	180-210	TACTCGTAGGGCAGGCTGCCTG	GAGACCTCAGGGTTGGTGATCAG
3	SPS0113	FAM	220-260	AAAGTGACACAACAGCTTCTCCAG	AACGAGTGTCCTAGTTTGGCTGTG
4	INRA0005	HEX	120-160	TTCAGGCATACCCTACACCACATG	AAATATTAGCCAACTGAAAACTGGG

Table 2. sequences of ISAG loci and primers for the MIX2 (panel locus multiplex2).

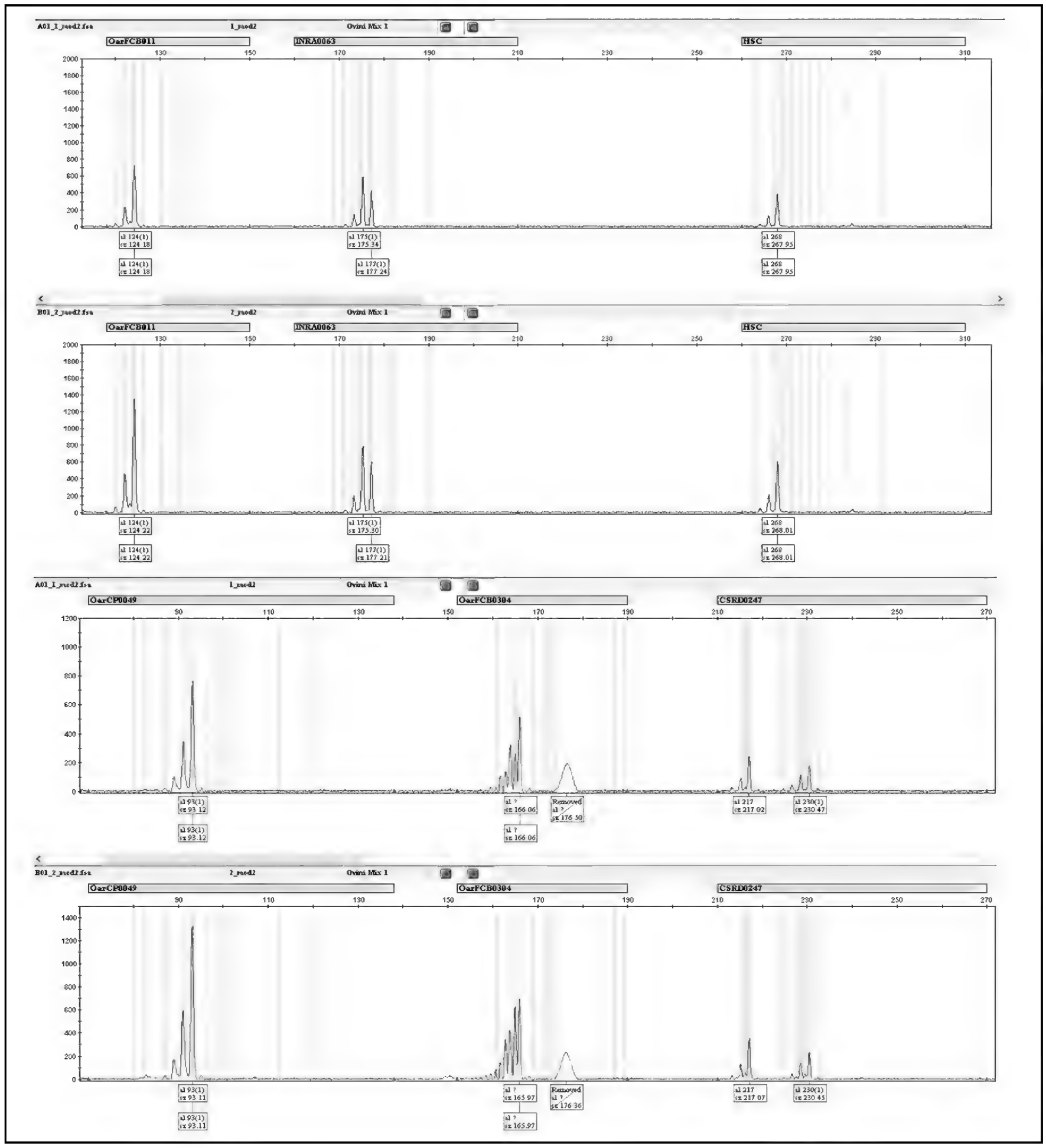

Figure 1. Electropherograms obtained during the genetic tests on Sicilian ovines.

RESULTS AND CONCLUSIONS

In figures 1, 2 some representative examples of electropherograms obtained during the genetic tests are shown.

These panels were obtained for all the samples examined and were used to collect polymorphic patterns based on STR. The colors indicate the various markers (FAM and HEX). All these data were tabulated for subsequent statistical processing.

At the moment the following objectives have been achieved:

- Optimization of the analytical method.
- Standardization of the use of selected markers.

These steps led to the preliminary analysis of allele frequencies and the database for the Sicilian sheep is currently being constructed by loading all the genotypes for each locus in the GeneAlex database in order to obtain the most common alleles. Expected heterozygosity data according to Hardy-Weinberg law and obtained heterozygosity. All this could be characterized by the autochthonous breeds and serves to construct the matrix of genotypes in terms of allele frequencies, used in forensic genetics for the attribution of possible kinships between the sheep.

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Comparative Biometrics of a Teleost Fish, *Boops boops* (Linnaeus, 1758) (Perciformes Sparidae) of the Algerian coast lines

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ABSTRACT Algeria is a country characterized by its diversified ichthyological fauna of economic and ecological importance, which deserves to be valorized by a scientific study. Due to this, our study is dedicated to the comparative biometry of the *Boops boops* (Linnaeus, 1758) (Perciformes Sparidae), between seven sites located on the Algerian coastline from north-east to north-west: El-kalla, Annaba, Skikda, Collo, Jijel, Algiers, Mostaganem. This study is made due to the total absence of reliable and exploitable information concerning the morphometric and meristic characteristics of this Algerian coast fish. The comparative study was carried out using thirty-six morphometric and meristic variables. The analytical approach carried out shows that environment factors have an influence and effect, not only on the diversity of living beings, but on the morphological variation in the same species. In addition, the statistical approach allowed a spatiotemporal evaluation of the biometry of the *B. boops* from the seven sites. As a first step, all the univariate statistical analyzes carried out, suggest significant differences between the seven sites, as well as a possible sexual dimorphism. Also, the analysis of variance at a fixed model classification criterion shows, with respect to the site factor, very significant to very highly significant differences between the seven sites for thirty variables out of thirty-six; for the sex factor, there are no significant differences for thirty-two variables out of thirty-six. Other models have been studied. Thus, in general, the general linear model MANOVA confirms the results obtained by the ANOVA.

KEY WORDS *Boops boops*; Mediterranean Sea; Algerian coast lines; ANOVA; MANOVA.

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INTRODUCTION

The biology or morphology of *Boops boops* (Linnaeus, 1758) (Perciformes Sparidae) has been studied in many areas: Tunisian coasts (Anato & Ktari, 1986), Moroccan (Zoubi, 2001a, b; Zoubi et al., 2004), Spanish (Zuniga, 1967), northwestern Mediterranean (Trangridis & Filippouzis, 1991; Sanchez-Velasco & Norbis, 1997), and Greece (Karpouzi et al., 2000). In Algeria, the study of the biology of the fish is limited in some works we quote that were carried out on the coasts of Bou-Ismail (Chali-Chabane, 1988) and Béni-Saf (Djabali et al., 1991). On the coast of Skikda, data on the bi-

ology or biometry of the *B. boops* fish and its ecology are missing.

As a result, our study attempts to answer a need for information on the *B. boops* biometrics of the Algerian coast, by comparing its biometric parameters of the samples taken from seven sites located on the Algerian coastline from East to West: El-Kalla, Annaba, Skikda, Collo, Jijel, Algiers, and Mostaganem. The biometric study is based on statistical processing of morphometric and meristic variable data measured on samples of the fish.

MATERIAL AND METHODS

The biometric study is based on samples of the *B. boops* fish taken from seven sites located along the Algerian coastline from north-east to north-west: El-kalla, Annaba, Skikda, Collo, Jijel, Alger, and Mostaganem.

A sample of 30 individuals (from 2.5 to 4 kg) is taken into consideration in each site, respecting as much as possible all size classes present. The details are given in Table 1. Each individual is wrapped in plastic film immediately after collection to avoid damage, and is put in the freezer at -20 °C. In the laboratory, a series of 36 morphometric and meristic measurements are made on each fish (Fig. 1).

Any statistical study can be broken down into at least two phases: the collection or collection of data, on one hand, and their analysis or interpretation, on the other.

Data collection has been dealt with in the previous paragraph. As for statistical analysis, it can be broken down into two stages, one deductive or descriptive and the other inductive.

The purpose of descriptive statistics is to measure and present the observed data in such a way that it can easily be seen, for example in the form of tables or graphs.

Statistical inference makes it possible to study or generalize under certain conditions the conclusions thus obtained by means of statistical tests by taking certain risks of error which are measured using the theory of probabilities.

For our work, all the calculations were performed for each variable and for each of the 7 sites, using a DELL-type microcomputer and using the Minitab version 16.1 statistical analysis and statistical processing software (X, 2011).

Univariate statistical analyzes

Description of the data. To better describe the different characteristics obtained by site, we calculated some basic statistical parameters such as the arithmetic mean (x), which is a parameter of central position and trend, the standard deviation (s), which measures the dispersion of the data around the mean, the minimum (x_{min}) and maximum (x_{max}) values which both give an idea of the extent of the data, and finally the size (n) which informs us about the importance of the data processed.

Cross-site comparison of average characteristics: Variance analysis test. To compare the averages for each of the 36 characteristics among the seven sites, we used the one-way variance analysis test or the fixed model classification factor. This test consists in comparing the averages of several populations from random, simple, and independent sample data (Dagnelie, 1970, 2006).

The test is performed either by comparing the value of F_{obs} with the corresponding theoretical value $F_{(1-\alpha)}$, extracted from the Fisher's F-table for a significance level $\alpha = 0.05$ or 0.01 or 0.001 and for k1 and k2 degrees of freedom, either by comparing the value of the probability p with always the

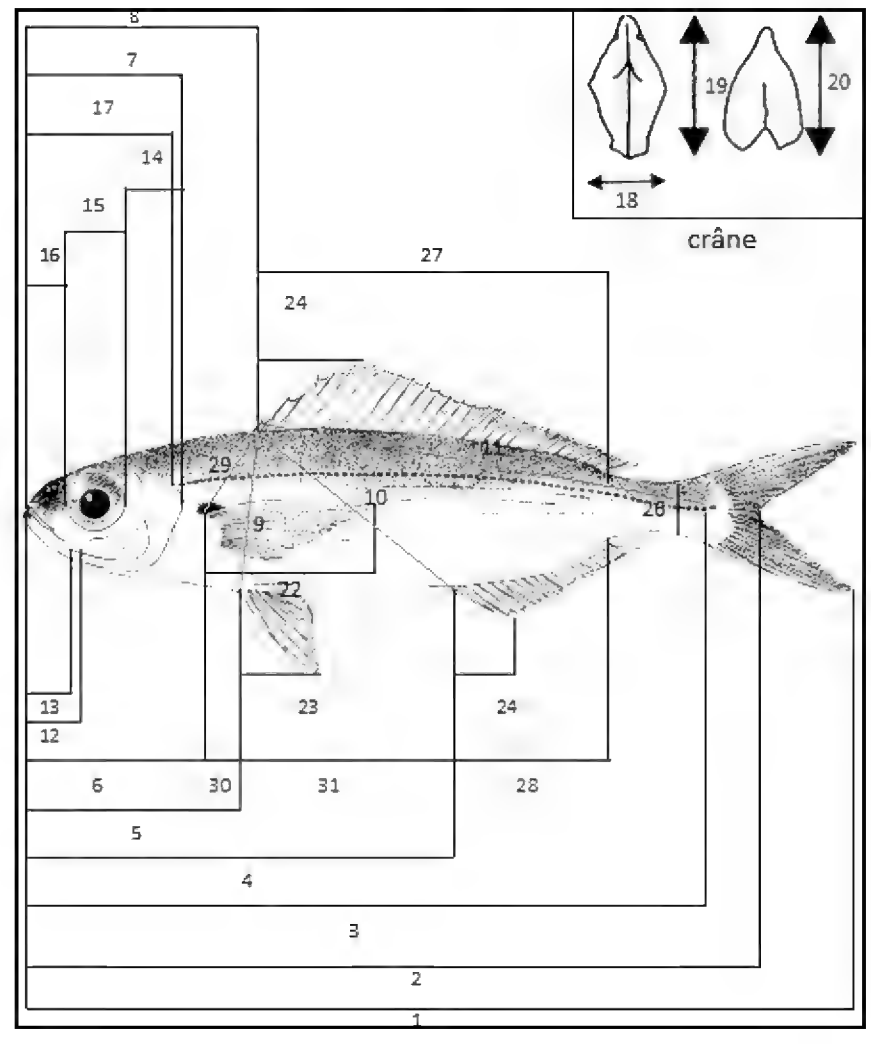

Fig.1 : Morphometric measurements made on each fish.

different values of (α), for values α = 0.05 or α = 0.01 or α = 0.001 (Mezedjri & Tahar 2007; 2008a, 2008b).

Depending on whether this hypothesis of equality of means is rejected at the level α = 0.05, 0.01 or 0.001, it is conventionally said that the difference observed between the means is significant, highly significant or very highly significant. These differences of one, two or three asterisks are generally marked (Dagnelie, 1970, 2006).

This test was used to compare, on one hand, between the seven sites, the averages of each of the 36 variables and, on the other hand, to compare between the two sexes in each site, the averages of the 36 presumed variables (Mezedjri & Tahar, 2008a, b).

Calculations are made using the Minitab software GLM procedure (X, 2011) for each of the 36 variables at the 7 sites.

Multivariate statistical analyzes

Comparison between sites for all characteristics: multivariate MANOVA variance analysis test. The comparison between the 7 sites for all 36 variables studied and between the two sexes in the 7 sites for all the variables measured, was performed by using multivariate analyses of variance using three statistical tests, that are: Wilk's lambda, Lawley-Hotteling, and Pillai's trace (Dagnelie, 1970, 1986, 2006).

This method is an extension of the univariate variance analysis, in which we have several variables that were observed simultaneously on the same individuals (or sites). The three tests cited above and proposed by Palm (2000) and Dagnelie (1970, 2006) are all asymptotically equal in power, and no test can be recommended in a systematic way, preferably to others (Dagnelie, 1986). According to Huberty (1994), the Wilk's test is the most popular.

Morphometric variables		
Number	Code	Description
1	Lt	Total length
2	Lf	Fork length
3	Ls	Standard length
4	Lpan	Pre-anal length
5	Lppv	Pre-pelvic length
6	Lppc	Pre-chest length
7	Lcep	Cephalic length
8	Lpdo	Pre-dorsal length
9	Dopv	Dorsal / pelvic distance
10	Doan	Dorsal / anal distance
11	Doca	Dorsal / caudal distance
12	Lman	Length of the mandible
13	Lmax	Maxillary length
14	Poor	Distance poste orbitaire
15	Dor	Orbital position distance
16	Pror	Pre-orbital distance
17	Lpop	Preoperative length
18	Lain	Inorbital width
19	Lcra	Skull length
20	Mist	Mandible / Isthmus length
21	Lapc	Distance between pectoral insertions
22	Hpc	Height of the pectoral
23	Hpv	Height of the pelvic
24	Hdo	Height of the dorsal
25	Han	Anal height
26	Hpdc	Height of the peduncle
27	Bado	Base of the dorsal
28	Baan	Base of the anal
29	Dopc	Dorsal / pectoral distance
30	Pcpv	Pectoral / pelvic distance
31	Pvan	Pelvic / anal distance
32	Cæc	Number of pyloric caecum
33	Brin	Number of lower gill rakers of the 1st left branchial arch
34	Brsu	Number of upper gill rakers of the 1st left branchial arch
35	Ryp	Number of rays of the left chest
36	Ryp	Number of left pelvic rays

Table 1. Morphometric and meristic variables studied.

Sites	Males			Females		
	n	Lt min	Lt max	n	Lt min	Lt max
El-kala	33	13.20	18.20	5	14.60	16.60
Annaba	16	15.30	24.70	10	14.60	24.50
Skikda	26	17.20	22.70	15	15.70	21.50
Collo	30	14.00	21.00	4	15.80	19.00
Jijel	19	13.80	21.30	21	14.40	22.00
Alger	17	14.80	21.30	21	14.50	21.90
Mostaganem	13	13.70	17.80	30	14.80	19.00

Table 2. Description of the data for each site.

RESULTS AND DISCUSSION

Results of univariate statistical analyzes

Calculation of basic statistical parameters. To better describe the different variables that characterize the individuals studied in two different sites,

we calculated some basic statistical parameters such as the arithmetic mean (x) which is a parameter of central position and trend, the difference type (s), that measures the dispersion of the data around the mean (x), the minimum values (Lt_{min}) and maximum values (Lt_{max}), which both give an idea of the extent of the data, and finally inform us about the size and the importance of the collected samples.

The biometric study was conducted using *B. boops* samples taken from seven different sites on the Algerian coast. We have inventoried a total of 261 individuals including 154 males, of which their total length Lt varies between 24.70 cm and 13.20 cm, 106 females, of which their Lt varies between 24.50 cm and 14.40 cm, and only two fish of indeterminate sex (Table 2).

It is noted that the total length Lt of the males is greater than that of the females for the sites of the gulf of El-kalla, Annaba, Skikda, and Collo whereas for the sites of the gulf, Jijel, Mostaganem, and the bay of Algiers it is reversed.

MANOVA multivariate analysis of variance

The analysis of variance has several variables or dispersion analysis, essentially to compare the averages of more than two populations for several variables (Dagnelie, 2000).

This is an extension of the univariate variance analysis, in which we have several variables that were observed simultaneously on the same individuals. Dagnelie (2000) and Palm (2000) provide several tests to perform the multivariate analysis of variance which are: the Wilk's Lambda test, Pillai's Trace, and Lawley-Hotelling. However, all these tests are asymptotically equal in power, and no test can be recommended in a systematic way, in preference to others (Dagnelie, 2000). According to Huberty (1994), the Wilk's test is the most popular.

The Minitab MANOVA command applied to the data of the two sites to perform multivariate variance analysis has two fixed classification criteria and whose sex factor is hierarchical at the site factor gives the results of the two following Tables 4, 5.

CONCLUSIONS

The aim of this work was to study the biometry of the *B. boops* taken from seven sites located along

	Factor site		Factor sexe	
Variables	**Fobs**	**P**	**Fobs**	**P**
Lt	15.33	0.000 ***	1.92	0.067 ns
Lf	12.40	0.000 ***	1.63	0.128 ns
Ls	14.24	0.000 ***	2.17	0.038 *
Lpan	12.11	0.000 ***	1.85	0.079 ns
Lppv	12.49	0.000 ***	1.10	0.366 ns
Lppc	13.31	0.000 ***	0.58	0.772 ns
Lcep	9.02	0.000 ***	1.48	0.174 ns
Lpdo	11.34	0.000 ***	1.28	0.259 ns
Dopv	3.77	0.001 ***	1.04	0.407 ns
Doan	7.21	0.000 ***	1.47	0.177 ns
Doca	7.42	0.000 ***	2.01	0.054 ns
Lman	1.90	0.081 ns	1.12	0.350 ns
Lmax	5.93	0.000 ***	0.36	0.926 ns
Poor	9.19	0.000 ***	1.36	0.221 ns
Dor	0.35	0.910 ns	0.54	0.807 ns
Pror	8.57	0.000 ***	0.49	0.839 ns
Lpop	11.91	0.000 ***	0.74	0.640 ns
Lain	3.33	0.004 **	0.42	0.887 ns
Lcra	18.23	0.000 ***	0.52	0.818 ns
Mist	9.48	0.000 ***	6.97	0.000 **
Lapc	7.17	0.000 ***	1.11	0.356 ns
Hpc	14.77	0.000 ***	0.81	0.580 ns
Hpv	7.98	0.000 ***	0.96	0.461 ns
Hdo	3.60	0.002 **	1.01	0.426 ns
Han	3.13	0.006 **	2.22	0.033 *
Hpdc	3.94	0.001 ***	0.44	0.873 ns
Bado	11.61	0.000 ***	1.35	0.226 ns
Baan	16.33	0.000 ***	1.64	0.124 ns
Dopc	9.34	0.000 ***	0.62	0.736 ns
Pcpv	10.48	0.000 ***	1.98	0.059 ns
pvan	7.26	0.000 ***	2.05	0.050 *
Cæc	23.37	0.000 ***	0.29	0.958 ns
Brin	12.79	0.000 ***	0.48	0.851 ns
Brsu	3.98	0.001 ***	0.67	0.701 ns
Rypc	16.11	0.000 ***	0.85	0.544 ns
Rypv	2.96	0.008 **	1.30	0.249 ns

$p > \alpha = 0.05$: (ns) not significant differences
$p \leq \alpha = 0.05$: (*) just significant differences
$p \leq \alpha = 0.01$: (**) highly significant differences
$p \leq \alpha = 0.001$: (***) very highly significant differences
ddl: degrees of freedom
SCE: sum of squared deviations
CM: middle square
Fobs: F value of Fisher.

Table 3. Results of the analysis of variance at a fixed model classification criterion of the comparison, between sites and sexes (sites), of the means of each of the 36 variables.

Tests	Observed value of the test	Fobs	DL	P
Wilks'	0.02235	5.191	216; 1247	0.000 ***
Lawley-Hotelling	5.94605	5.707	216; 1244	0.000 ***
Pillai's	2.64014	4.671	216; 1284	0.000 ***
$p \leq \alpha = 0{,}001$: (***) very highly significant differences				

Table 4. MANOVA for Sites. Multivariate tests used to test the equality of mean vectors between sites.

Tests	Observed value of the test	Fobs	DL	P
Wilks'	0.28418	1.155	252; 1451	0.062ns
Lawley-Hotelling	1.40953	1.159	252; 1451	0.057ns
Pillai's	1.13044	1.150	252; 1505	0.067ns
$p > \alpha = 0{,}05$: (ns) differences not significant				

Table 5. MANOVA for Sexes (Sites). Multivariate tests used to test the equality of mean vectors between the two sexes in the sites.

the Algerian coastline from north-east to north-west: El-kalla, Annaba, Skikda, Collo, Jijel, Alger, Mostaganem.

The comparative biometric study between the seven sites showed that the application of the generalized linear model or the analysis of the variance to a criterion of ANOVA fixed model classification carried out for each of the 36 variables measured to compare between the seven sites and between the two sexes, showed that:

• Regarding the site factor, we find that there are highly to very highly significant differences for 34 out of 36 variables. The 4 variables where the differences are highly significant are: Rypv, Lain, Hdo, and Han, the 2 variables that do not show significant differences are: Dor and Lman.

• For the sex factor, there are no significant differences for 32 out of 36 variables. Variables with significant differences at the $\alpha = 5\%$ level are: Ls, Han, and Pvan. The Mist variable (mandible-isthmus) has very highly significant differences at the $\alpha = 0.1\%$ level.

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Breeding populations of *Bombina orientalis* Boulenger, 1890 (Amphibia Anura Bombinatoridae), in a degraded urban habitat in Vladivostok, Russia

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ABSTRACT Most amphibian species are declining, and while the causes of such decline are multiple, environmental pollution is one of the most important. *Bombina orientalis* Boulenger, 1890 (Amphibia Anura Bombinatoridae), is known to be sensitive to pollution. However, we report here that the species breeds in highly polluted water within the city of Vladivostok, Russia. The species was found at the same site for two consecutive years, although in lower numbers in the second year. The resilience of *B. orientalis* to pollution is not consistent with other populations within the range of the species, and is therefore important in the frame of conservation.

KEY WORDS *Bombina orientalis*; breeding habitat; urbanisation; degraded habitat; Russia.

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INTRODUCTION

Amphibian (Amphibia Anura) populations are on the decline (Stuart et al., 2004; Pimm et al., 2014), and cities or urban areas are principally devoid of their presence. Most amphibian species require pristine and well connected habitats (Collins & Storfer, 2003; Becker et al., 2007), and are not present in ecologically disturbed areas.

This is, for instance, the case of the *Rana* Linnaeus, 1758 (Amphibia Anura Ranidae), species in North America, the genus with the highest number of negative responses to urban habitats (Scheffers & Paszkowski, 2012). Only a few species are able to withstand the degraded ecological conditions of cities or urban areas as well as *Duttaphrynus melanostictus* (Schneider, 1799) (Bufonidae), a species commonly found in degraded areas and cities (Holzer et al., 2017).

One of the problems of urbanised areas is the high degree of pollution, besides the absence of adequate habitat, as environmental contamination negatively impacts amphibian populations (Sparling et al., 2010; Egea-Serrano et al., 2012). The effects of pollution are multiple, and resulted for instance in the decline of *Acris crepitans* Baird, 1854 (Hylidae) (Reeder et al., 2005), and in the absence of *Dryophytes suweonensis* (Kuramoto, 1980) (Hylidae) at sites with high pollution levels (Borzée et al., 2018).

Bombina orientalis Boulenger, 1890 (Bombinatoridae), the Oriental fire-bellied toad, is a species ranging from the Korean and Shandong peninsulas in the South to about 125° North in China. Is it how-

ever common in Primorsky Territory in Russia up to 135° N, and present in small isolated groups in Khabarovsk Territory, up to 136° N. *Bombina orientalis* is monophyletic within its range, despite local genetic and morphological variations (Kuzmin et al., 2010; Fong et al., 2016), with two morphological forms described in Primorsky Territory, Russia (Korotkov, 1972). The mostly terrestrial variety "*sylvatica*" is usually associated with forest habitats and never present in open wetlands further than 200 m from the forest border (Kuzmin & Maslova, 2005), while the aquatic variety "*praticola*" is only recorded in sedge and reed meadows when associated with grasslands (Korotkov, 1972). The species is known to be sensitive to environmental pollutants due to abnormal development when close to human activities (Kang et al., 2016) and failure of embryonic development in polluted waters (Park et al., 2014). The species is common in forested zones around Vladivostok, Russia (Kuzmin & Maslova, 2005), but was not expected to be present within the city due to habitat disconnection and heavy pollution (Vshivkova et al., 2014 ; Vshivkova, 2016; Maslova et al., 2016). It should, however, be noted that *B. orientalis* was observed in some forested parts of the city about 100 years ago; when the city was c. 50 year old (Kuzmin & Maslova, 2005). The situation deteriorated in the 1950s and 1960s, when the city was actively built up, especially in the focal areas of this study. This study aim at documenting the continued breeding activity of *B. orientalis* in polluted areas, within the city of Vladivostok.

MATERIAL AND METHODS

Vladivostok is situated on the Muravyov-Amursky Peninsula, surrounded by the Sea of Japan and was originally completely covered by forests of cedar and broadleaved trees (Avdeev, 2015). Because of the peninsular location of the city, all urban streams are, at present, isolated from natural biotopes and are heavily polluted, following the industrial development of the city. Only *Rana dybowskii* Günther, 1876 (Ranidae), was known to occur within the northern part of the city, although several amphibian species are present in small isolated populations on its outskirts, including *B. orientalis* (Maslova et al., 2016). The study area, Cape Firsov, is sandwiched between the city and the coastal line, preserved because of a railway, and mainly used for housing, warehouses, landfills, and wastelands (Fig. 1). The vegetation of the area is composed of individual willows and elms. A pond is present in the area, used for landfills and sewages dump.

Surveys and data analysis

We conducted aural and visual surveyed in Cape Firsov on 17 May 2016 to assess for the presence of *B. orientalis*. We annotated abiotic variables, vegetation, and pollution status at the site during the survey. To assess the presence of a breeding population, we conducted the same surveys on 27 April 2017 and again on 4, 18, and 19 August 2017 and annotated the presence of adult and juvenile amphibians present at the site. Environmental pollutants were also recorded by types. Our data subsequently qualitatively analysed the occurrence of the focal species and environmental pollutants.

RESULTS

Here, we report the first observation of *B. orientalis* within the city of Vladivostok, surviving in a highly polluted environment, and completely isolated from other populations. The first survey on 17 May 2016 resulted in the detection of multiple calling individuals (air temperature = 19.3 °C) within plastic bags in a ditch located 80 meters east of the pond and 200 meters from the shore (Fig. 1). The ditch was littered with rubbish, mostly plastic bottle and bags, but also other heavy household wastes, and covered by herbaceous vegetation, with maximum water depth of 30 cm (Fig. 2). An individual was helped out of a bag to visually confirm the species.

Bombina orientalis was detected during the surveys conducted in 2017 to confirm the continuous presence of the species at the site over years, although seemingly impacted by local conditions. On 27 April 2017, no individuals were found: the ditch were the individuals were found the year before was dry. The trash included plastic bags and bottles, as in 2016, but also tyres and heavy metal parts. Besides, the dump had been used for burning, and the ground was scorched around the ditch (Fig. 3). Two adults were detected at the pond, despite the pol-

Figures 1,2. Site where *Bombina orientalis* was found breeding in Vladivostok in 2016. The site is isolated from other populations by the sea on three sides, and the city on the other one.
Figure 3. Site where *Bombina orientalis* was found breeding in Vladivostok, Russia, in 2016. The same type of trash was found in 2016 and 2017, with the addition of tyres and heavy machinery parts in 2017. Besides, the dump site had been burnt in 2017.

luted conditions, on 4 August 2017, a single juvenile was found in the vicinity of the pond on 18 August 2017, and finally, two adults and one juvenile were found on 19 August 2017, at the same location. The presence of juveniles highlights the breeding activity of *B. orientalis* at the site, despite the heavy pollution.

When comparing for the type of environmental pollution, the sewage dump was the same at the pond in 2016 and 2017, but we did not detect the species in the pond in 2016. The land pollution mostly comprised plastic items in 2016 and 2017, with the addition of burnt items and tyres plus heavy machinery parts in 2017. The species was found breeding in the ditch in 2016 only.

DISCUSSION AND CONCLUSIONS

Our observations first demonstrate the presence of *B. orientalis* within the city of Vladivostok in Russia. We also demonstrate that the species can breed, at an unknown fitness cost, in highly polluted and disturbed conditions. These observations therefore hint at an unexpectedly high ecological plasticity in *B. orientalis*. Furthermore, based on the presence of morphological abnormalities close to cities (Kang et al., 2016), we could have expected individuals with additional or missing limbs, which was not the case. We expect the hatching and development rate of individuals breeding in the pond to be significantly lower, as Park et al. (2014) reported the failure of embryonic development in polluted waters. Finally, we hypothesise that calling individuals found in 2016 were in the plastic bags as they would have provided a warmer environment, and may have potentially provided call amplifiers.

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Research and identification of *Staphylococcus* Pasteur, 1880 (Bacillales Staphylococcaceae), potentially zoonotic, isolated from Sicilian dogs

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ABSTRACT The uncontrolled abuse of antibiotics used in veterinary medicine, has led to the development of some mechanisms of antibiotic-resistence in the bacteria. This event allows them to breed and increase in number inside a host organism. *Staphylococcus* spp. strains (Bacillales Staphylococcaceae) have been isolated from cutaneous swabs of dogs, have been identified through microbiological methodologies on a biochemical basis, and their sensitive profile to various antibiotics, commonly used in the veterinary domain and in human medicine, was valued. Other molecular and microbiological studies on these *Staphylococcus* spp. strains have also been carried.

KEY WORDS *Staphylococcus*; Sicilian dog; antibiotic-resistence.

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INTRODUCTION

In the last decades, as a result of the uncontrolled abuse of antibiotics used in veterinary medicine, bacteria have developed some mechanisms of antibiotic-resistence, which allow them to breed and increase in large numbers inside a host organism. In spite of this, pharmaceutical industries continue to launch new types of antibiotic drugs on the market, as soon as new resistances are developed.

Staphylococcus pseudintermedius Devriese, Vancanneyt, Baele, Vaneechoutte, De Graef, Snauwaert, Cleenwerck, Dawyndt, Swings, Decostere et Haesebrouck, 2005, is positive Gram's cocci, that commonly lives inside pets, where it colonize the oral cavity, the nasal mucosa, the abdominal skin, and the arial mucosa, causing abscesses, purulent infections, pyometra, folliculitis, dermatitis, and otitis externa as well (Devriese et al., 2005; Weese & Van Duijkeren, 2010).

Since 1990, the emergence of the *Staphylococcus pseudintermedius* strains, resistant to methicillin (MRSP), has been identified in infections of a huge number of species of pets and other animals. These MRSPs have often been isolated from dog specimens, shifting the scientific community attention on pets, as a potential source of resistant bacteria, which are transmissible to humans (Devriese et al., 2005; Bannoehr & Guardabassi, 2012).

The aim of this work is the multidisciplinary study of *Staphylococcus* spp. strains, isolated from cutaneaus swabs of dogs. The isolated strains have been identified through microbiological methodologies on a biochemical basis, evaluating their sensitive profile to various antibiotics, commonly used

in the veterinary domain, and which find similar molecules in human medicine.

A molecular analysis results for some strains were compared with those obtained by the classical microbiological methods.

Furthermore, rDNA sequences obtained for various species were studied to reveal differences and genetic distances in the isolated strains. The sequences were compared in the Wu Blast database.

MATERIAL AND METHODS

Sampling

During the study period, 275 swabs were taken from infected animals, 65 of them were positive to the growth of *Staphylococcus* spp.

Microbiological analysis

Each swab sample was employed for the bacterial isolation on Mannitol Salt Agar, that it is selective for *Staphylococcus* spp.

Staphylococcus positive samples were identified by Gram staining (*Staphylococcus* Gram positive), following: catalase test (*Staphylococcus* catalase positive), oxidase test (*Staphylococcus* oxidase negative), coagulase test (*Staphylococcus* coagulase positive), Voges-Proskauer test *S. aureus* Rosenbach, 1884 (VP +), and *S. intermedius* Hájek 1976 (VP-) tests, STAPH API test used to identify the species.

All the isolated bacterial strains were subjected to an exam to evaluate their sensitivity resistence and intermediate reactivity to antibiotics.

Subsequently, the following antibiotics were tested: amoxicillin, enrofloxacin, marbofloxacin, convenia, methicillin, doxyciclyne, penicillin, and pradofloxacin. These antibiotics are all commonly used in veterinary medicine.

Molecular analysis

Molecular biology investigations were performed by extracting the DNA. The PCR was targeted to a specific 16S rRNA gene fragment 600 bp long. The amplicones were loaded on agarose gel to confirm the PCR results. The samples of amplified DNA were purified with a commercial kit "GFX PCR DNA and gel band purification kit", the sequence products were placed in a filtering column containing a particular "Sephadex®" resin using the "Illustra" purification kit AutoSeq G-50 Dye. Sanger sequencing allowed to obtain a sequence of a nucleic acid molecule by identifying the samples on a molecular basis.

Twenty-one strains of S*taphylococcus* spp. were selected for the molecular proof: the results obtained after the sequencing procedure were compared with recorded sequences for the identifications with the online system BLASTn on the GenBank database and then aligned through the Geneious program.

RESULTS AND DISCUSSION

On the base of the microbiological and biochemical tests were obtained the following identifications: n° 38 strains of *S. aureus*, 18 *S. xylosus* Schleifer et Kloos, 1975, 5 *S. lentus* Kloos, Schleifer et Smith, 1976, 2 *S. hycus*, 1 *Staphylococcus lugdunensis* Freney et al., 1988, 1 *S. epidermidis* (Winslow et Winslow 1908) Evans, 1916. Thanks to the analysis done with the VP test: 38 strains were identified by the API system as *S. aureus*: 20 were identified as *S. intermedius* (negative VP).

In line with what previously described by other authors, that associates a greater probability to find a colonization done by *Staphylococcus pseudintermedius* in samples coming from animals (Futagawa-Saito et al., 2006), the VP test allowed to differentiate and identify 30% of the strains as *S. pseudintermedius* against 27% of *S. aureus*. Moreover, the data obtained from the antibiograms show that 3 staphylococcal species have a strong resistence to methicillin and in particular 88% of the *S. aureus* are MRSA, 63% of the *S. pseudointermedius* are MRSP, and only 16% of *S. xylosus* show a resistenace to methicillin. These data confims the recent emergence regarding the MRSP strains diffusion in veterinary field (Perrent et al., 2010).

The antibiograms performed on all the strains of isolated staphylococci, allow us to understand the different degree of resistence/sensitivity to the antibiotics most commonly used in veterinary medicine, as well as in the human domain. So our study

confirms the emergence of the phenomenon of resistence to antibiotics also in the veterinary field, paying particular attention to the high presence of penicillin-resistant strain (90%), methicillin (47.7%), and amoxicillin (27.7%). The data obtained show a strong sensitivity to difluoroquinolones in a higher percentage than the one cited in a previous literature (Humphries et al., 2016; Yarbrough et al., 2018).

As a matter of fact, in the veterinary field, this last class of antibiotics is primarily used for the infections caused by *S. pseudintermedius* (Perreten et al., 2010).

The molecular analysis used as support to the traditional identification techniques have confirmed the identification of isolated staphilococci species. On 17 strains, it has not been possible to confirm strains of *S. pseudointermedius*.

The molecular identification results, obtained in this work, are in line with what is reported in the bibliography (Bannoehr & Guardabassi, 2012) and underline the presence of potentially pathogenic

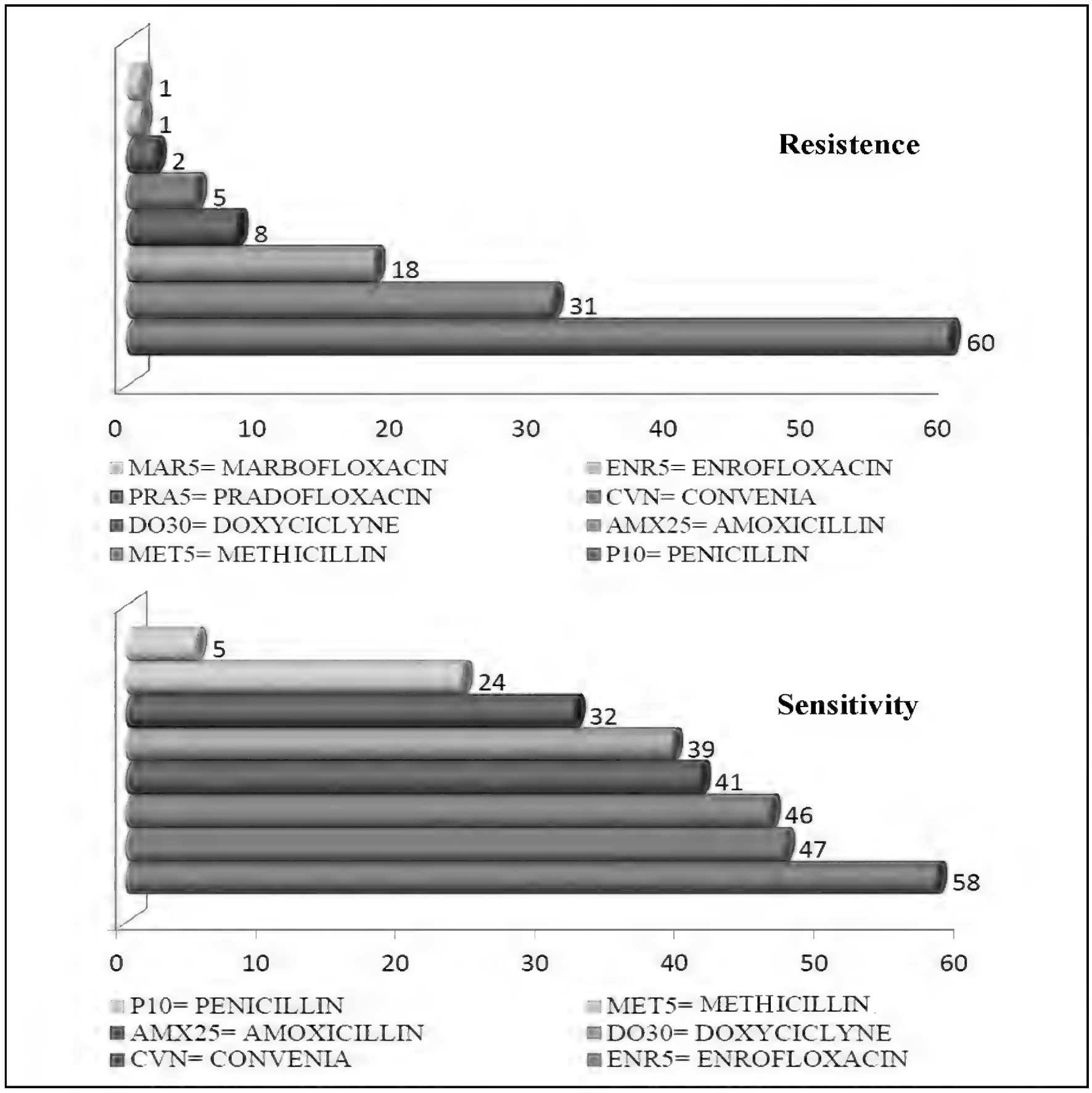

Figure 1. Number of isolated strains for which was detected the antibiotic resistance panel.

positive coagulase strains belonging to the *S. aureus* and *S. pseudintermedius* in pets, and in particular in dogs. The 16S rDNA sequence data allowed us to analyze the genetic distance in the isolated strains and those in the GenBank.

The increasing feasibility of "high-throughput" sequencing suggests that it is promising as a rapid procedure to differentiate a number of pathogens in a biological sample.

This work is proposed as the continuation af a surveillance program, started in 2012, on 100 samples taken from dogs, only 2 of them were detected as resistant to methicillin.

In this work, 20 of 65 isolates were identified as *S. pseudointermedius*, 13 of which were MRSP. Thirty-one strains showed resistance to methicillin. The validated approach could be employed on a large scale for epidemiological studies in regions where antibiotic resistances are very diffused.

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Bioacoustic evidence of two uncommon crickets from SW Sardinia, including an analysis of the song of *Brachytrupes megacephalus* (Lefèvre, 1827) (Orthoptera Gryllidae) in the ultrasonic range

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ABSTRACT Two elusive species of crickets, *Natula averni* (A. Costa, 1855) (Orthoptera Gryllidae Trigonidiinae), and *Brachytrupes megacephalus* (Lefèvre, 1827) (Orthoptera Gryllidae Gryllinae) are reported from SW Sardinia based on recordings with 96 kHz sample frequency. The song of the latter species, that was observed and photographed during song emission, is also recorded at a sample frequency of 250 kHz, revealing harmonic components up to 100 kHz and above.

KEY WORDS Orthoptera; *Brachytrupes*; *Natula*; Sardinia; Biogeography; Bioacoustics.

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INTRODUCTION

Previous bioacoustics studies by the author in the same geographic area (Brizio & Buzzetti, 2014; Brizio, 2015) have demonstrated the effectiveness on the field of low cost USB microphones capable of recording ultrasounds thanks to high sampling frequency (250 kHz), the consistency of such recordings with those obtained with a 96 kHz audible band digital recorder, and the possibility to identify different species on the basis of their song, as well as to derive novel information from an analysis of high frequency components of the songs, previously unreported in the scientific literature: an observation limited to audible frequencies, or to the 0-48 kHz range provided by 96 kHz recordings, may deliver incomplete or misleading results.

In this short paper, the same techniques cited above are applied to the bioacoustic identification and to the analysis of the song of two elusive species of Orthoptera Gryllidae: *Natula averni* (A. Costa, 1855), also described as "*the most mysterious orthopteran of Europe*" (Odé et al., 2011), and the biggest European cricket, *Brachytrupes megacephalus* (Lefèvre, 1827), capable to emit an exceptionally powerful song. Although lacking field equipment to measure absolute acoustic pressure, based on the direct experience of close contact with both species, the author subjectively feels that the volume of the song of *B. megacephalus* matches or exceeds the volume of the songs by *Gryllotalpa vineae* Bennet-Clark, 1970, the latter species reportedly reaching 80 dB at 2 meters above the burrow (Bennet-Clark, 1970a, b).

In the following pages, the analysis of the song of *B. megacephalus* will reveal a previously unreported, exceptionally rich harmonic structure, extending well into the ultrasonic domain.

MATERIAL AND METHODS

To ensure consistency with previous analyses (Brizio & Buzzetti, 2014; Brizio, 2015), the same methods have been applied here.

All the species reported were recorded within a 10 km range from Fluminimaggiore (Carbonia-Iglesias Province, Sardinia, Italy) (Figs. 1–5).

All the audio material was obtained by field recording. Specimens were not captured nor recorded in constrained conditions.

Recording equipment included a Zoom H1 handheld digital Micro-SD recorder, coupled with a self-built stick stereo microphone using Panasonic WM-64 capsules from an Edirol R-09 digital recorder.

Acoustic recordings were taken in stereo, 16 bit, with a 96 kHz sampling frequency, and thus covering frequencies up to 48 kHz. The audio samples where obtained in the field, in windy conditions. Having observed no meaningful pattern in the lowest frequency range, to improve the clarity of the successive analyses and illustrations, an 18th order Chebychev Type 1 high pass filter was applied, with cut-off frequency at 150 Hz.

Due to atmospheric conditions, the left channel of the stereo microphone recorded a significant amount of noise. To the purpose of the analyses performed, the audio from the right channel was doubled in the left channel with no variation, before merging the two channels in one monophonic recording.

Ultrasound monophonic recording at a 250 kHz sampling frequency was performed via a Dodotronic Ultramic 250 microphone connected via USB cable to an Asus Eee PC 1225B notebook personal computer, using SeaWave software by CIBRA-University of Pavia's "Centro Interdisciplinare di Bioacustica e Ricerche Ambientali". Originally received as amplitude data (mV) by the recording apparatus, software normalized spectral energy is expressed in decibels. Sound pressure is expressed in dB Full Scale.

Oscillograms, spectrograms, and frequency analysis diagrams were generated by Adobe Audition 1.0 software. To give more evidence even to the faintest significant spectral components, or to improve readability of frequency/volume analyses, screenshots were contrast-enhanced with Adobe Photoshop Elements 4.0 by a procedure involving in sequence: color removal, image inversion, brightness and contrast adjustment, shadows/highlights adjustment. Those interventions did not affect the accuracy of rendering.

The illustrations of frequency analyses where generated with a scan of the entire audio sample

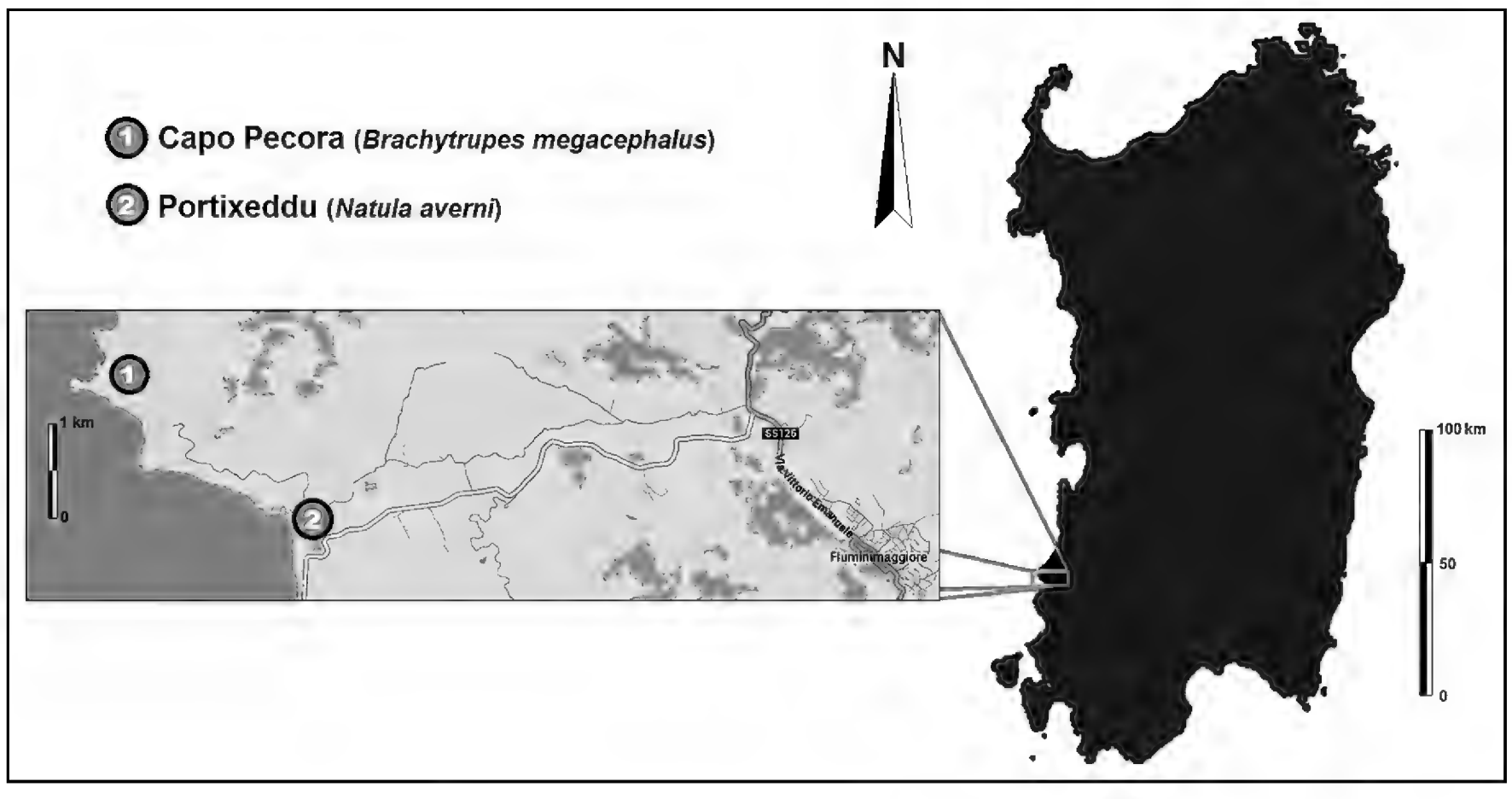

Figure 1. Map of the recording stations of Capo Pecora and Portixeddu, Sardinia.

(Table 1, column "Interval analysed") and with an FFT size of 4096 byte, capable to render a smoother and clearer picture. All the software cited operates under Windows 7 64-bit operating system.

Brizio & Buzzetti, 2014, address in more detail some technical requirements of Ultramic 250 and proposed a specific operating protocol to ensure comparability between Ultramic recordings and audio range recordings available in literature. Brizio (2015) describes in more detail the challenges of ultrasound field recording and the requirement to obtain a recording as near as possible to the singing animal, to avoid the attenuation of faint high-order harmonic components.

Audio samples examined	Duration (mm:ss)	Sampling frequency	Interval Analyzed in figures 6 - 16
B_megacephalus_C_Pecora_20180422-205622.wav	00:37	250 kHz	0:16:87 to 0:19:87
B_megacephalus_C_Pecora_20180422-205757.wav	00:18	250 kHz	not illustrated
B_megacephalus_C_Pecora_20180422-205423.wav	00:53	250 kHz	
B_megacephalus_C_Pecora_20180422-205208.wav	00:34	250 kHz	
B_megacephalus_CapoPecora_21_04_2018_A.WAV	00:35	96 kHz	0:21:00 to 0:26:00
B_megacephalus_CapoPecora_21_04_2018_A.WAV	00:21	96 kHz	not illustrated
Natula averni Portixeddu 24_04_2018 18_30 A.WAV	01:35	96 kHz	0:42:00 to 1:00:00
Natula averni Portixeddu 24_04_2018 18_30 B.WAV	03:19	96 kHz	not illustrated
Natula averni Portixeddu 24_04_2018 18_30 C.WAV	02:02	96 kHz	
Natula averni Portixeddu 24_04_2018 18_30 D.WAV	00:40	96 kHz	

Table 1. List of the audio samples examined in this paper. Also the recording whose analysis is not illustrated, were found consistent with the audio samples deetailed in this paper.

RESULTS

Systematics

Ordo ORTHOPTERA Burmeister, 1839
Familia GRYLLIDAE Laicharting, 1871
Subfamilia TRIGONIDIINAE Saussure, 1874
Genus *Natula* Gorochov, 1987

Natula averni (A. Costa, 1855)

EVIDENCE COLLECTED. Bioacoustical.

EXAMINED MATERIAL. Four 96 kHz recordings (Table 1) from Italy, Sardinia, Portixeddu (Carbonia-Iglesias Province), 24.IV.2018, 39°26'21.20"N - 8°24'46.84"E, 0 meter asl.

DISTRIBUTION. General distribution in Italy of this species, as reported by Odé et al. (2011) includes Campania (Averno: Costa, 1855; Targioni-Tozzetti, 1878). Schmidt & Hermann (2000) reported for Sardinia *Anaxipha longipennis* (Audinet-Serville, 1839), a species that, according to Odé et al. (2011), may be a synonym of *N. averni*. Boittier et al. (2006, 2007) reported *N. averni* from Corsica. For other data, see Massa et al. (2012).

REMARKS. Although unaided by visual recognition, due to the elusiveness of this very small species and to its habitat (reed vegetation along rivers near the sea, as confirmed by Odé et al., 2011), posing unsurmountable problems in the localization of this 1 cm long insect, identification poses no doubt, both for its habitat (Fig. 2) and for the unequivocal identification of the pattern and of

Peak number	Acoustic pressure dBfs	Peak frequency Hz	Theoretical Harmonic Frequency Hz	% difference with the Nth Harmonic
Fundamental	-25.03	6469	--	--
Secondary Peak	-57.95	12350	--	--
2nd Harmonic	-55.43	12980	12938	0.32%
Secondary Peak	-69.86	19170	--	--
3rd Harmonic	-67.69	19450	19407	0.22%
4rd Harmonic	-70.6	25940	25876	0.25%

Table 2. Numeric data from the frequency analysis of a 96 kHz recording of *Natula averni*, Portixeddu, 24.IV.2018.

Sample frequency	96 kHz				250 kHz				
Peak number	Acoustic pressure dBfs	Peak frequency Hz	Theoretical Harmonic Freq. (Hz)	% difference with the Nth Harmonic	Acoustic pressure dBfs	Peak frequency Hz	Theoretical Harmonic Freq. (Hz)	% difference with the Nth Harmonic	% difference with 5800 Hz Harmonics
Fundamental	-19.58	5812	--	--	-15.02	5920	--	--	--
2nd Harmonic	-27.78	11740	11624	0.99%	-19.65	11770	11840	0.59%	1.44%
Secondary peak	-60	~16300	--	--	-43.17	16350	--	--	--
3rd Harmonic	-54.64	17710	17436	1.55%	-43.92	17510	17760	1.43%	0.63%
Secondary peak	--	--	--	--	-40.59	23070	--	--	--
4th Harmonic	-64.85	23250	23248	0.01%	-40.42	24290	23680	2.51%	4.49%
Secondary peak					-58.76	27640			
5th Harmonic	-70.04	29360	29060	1.02%	-56.17	29050	29600	1.89%	0.17%
6th Harmonic	-76.11	35410	34872	1.52%	-61.57	34850	35520	1.92%	0.14%
7th Harmonic	-79.73	41100	40684	1.01%	-63.55	41070	41440	0.90%	1.14%
Secondary peak	-85	~42700	--	--	-64.81	42780	--	--	--
Secondary peak	--	--	--	--	-68.04	44730	--	--	--
8th Harmonic	-82.92	46920	46496	0.90%	-63.04	46560	47360	1.72%	0.34%
9th Harmonic	--	--	--	--	-64.52	52360	53280	1.76%	0.31%
10th Harmonic	--	--	--	--	-67.53	58220	59200	1.68%	0.38%
11th Harmonic	--	--	--	--	-69.28	64080	65120	1.62%	0.44%
12th Harmonic	--	--	--	--	-71.6	69760	71040	1.83%	0.23%
13th Harmonic	--	--	--	--	-74.24	75500	76960	1.93%	0.13%
14th Harmonic	--	--	--	--	-76	81350	82880	1.88%	0.18%
15th Harmonic	--	--	--	--	-77.43	87210	88800	1.82%	0.24%
16th Harmonic	--	--	--	--	-79.63	93200	94720	1.63%	0.43%
17th Harmonic	--	--	--	--	-80.08	98870	100640	1.79%	0.27%
18th Harmonic	--	--	--	--	-80.02	104670	106560	1.81%	0.26%
19th Harmonic	--	--	--	--	-80.41	110650	112480	1.65%	0.41%
20th Harmonic	--	--	--	--	-79.93	116390	118400	1.73%	0.34%
21th Harmonic	--	--	--	--	-77.09	121700	124320	2.15%	0.08%

Table 3. Numeric data from the frequency analysis of 96 kHz and 250 kHz recordings of *Brachytrupes megacephalus*, Capo Pecora, 21–22.IV.2018. Only the most relevant among the secondary peaks are considered in this table.

the harmonic structure well noted for the species and reported by Odé et al. (2011).

Figures 6–8 are self-explanatory and describe clearly the pattern and the structure of the song of *N. averni*, recorded at an air temperature around 22 °C. The song observed consisted of echemes lasting around 250 ms, regularly repeated at the rate of about 2/s. Echemes consist of about 10–20 syllables with a slight volume increase in the first four syllables, then equally loud. Frequency analysis in figure 8 and Table 2 shows the main volume peak at the fundamental frequency of around 6500 Hz, with three well-defined harmonics, the 2nd and the 3rd of which are preceded by a clearly discernible secondary peak.

The particular nature of the habitat, that made very difficult the handling of a portable computer, as well as the complete absence of any hint of high-order harmonic components in the 96 kHz recording, discouraged the attempt to record this species at a 250 kHz sampling frequency.

Subfamilia GRYLLINAE Laicharting, 1871
Genus *Brachytrupes* Serville, 1839

Brachytrupes megacephalus (Lefèvre, 1827)

EVIDENCE COLLECTED. Bioacoustical and photographic.

EXAMINED MATERIAL. Two 96 kHz and four 250 kHz recordings (Table 1) from Italy, Sardinia, Capo

Figure 2. The recording station of *Natula averni*, Portixeddu, 24.IV.2018. Figure 3. The recording station of *Brachytrupes megacephalus*, Capo Pecora, 21–22.IV.2018. Figure 4. A typical burrow of *B. megacephalus*, Capo Pecora, 21–22.IV.2018. Figure 5. Habitus of *B.megacephalus*, Capo Pecora, 22.IV.2018.

Pecora (Medio Campidano Province), 21–22.IV.2018, 39°27'3.27"N - 8°23'46.67"E, 20 meters asl. Air temperature around 19 °C / 20 °C.

DISTRIBUTION. It's the only Italian representative of the genus. General distribution in Italy of this species, as reported by Odé et al. (2011) includes Sicily as type locality, and Southern Sardinia (Zanardi, 1964; Baccetti, 1991; Schmidt & Hermann, 2000; Galvagni, 2010). However, the species is widespread in North Africa and is reported also from the Maltese islands. For other data see Massa et al. (2012).

REMARKS. Both the song and the habitus of this species are unmistakeable. Songs were recorded and specimens were observed on the promontory of Capo Pecora, delimited by granitic cliffs and boulders. In the plateau topping the promontory, the species burrows in the coarse sand resulting from the weathering of granite.

The burrows, the habitus, and the behaviour of the specimen, observed and photographed while singing, corresponded with the description by Odé et al. (2011).

The impressive looks of this cricket have no equal in the Italian fauna. The specimens, that sing during the spring, especially in April, emerge from their burrows at twilight and are very suspicious, ready to re-enter their den if disturbed. Even though the song can be heard from great distance and easily traced, to get the best recording and photographic opportunities, it is better to search for the dens and mark their position for successive visits, considering that the source of such a powerful song, subjectively perceived as reaching 80 dB at around 2m above and behind the animal, is uneasy to locate precisely.

Provided that any noise and rapid movements are avoided, the specimens can be illuminated and observed, in particular during the emission of the song, whose almost unbelievably high volume, with the physical excerption required, apparently saturates the perceptive faculties of the cricket, to the point when it can be lightly touched without escaping.

The pattern described by Odé et al. (2011) was observed: syllables continuously repeated at a rate of around 130/sec.

The recordings at 96 kHz and 250 kHz yielded consistent results, reported in Table 3.

In both the types of recording, the first seven "octaves", delimited by the fundamental frequency and the first seven harmonics, can be clearly made out in both the analyses (Figures 13, 14) and in both the time/frequency spectrograms (Figures 10, 12). Furthermore, as can be expected from an highly stressed sounding apparatus, secondary peaks and clusters of ancillary frequencies are generated.

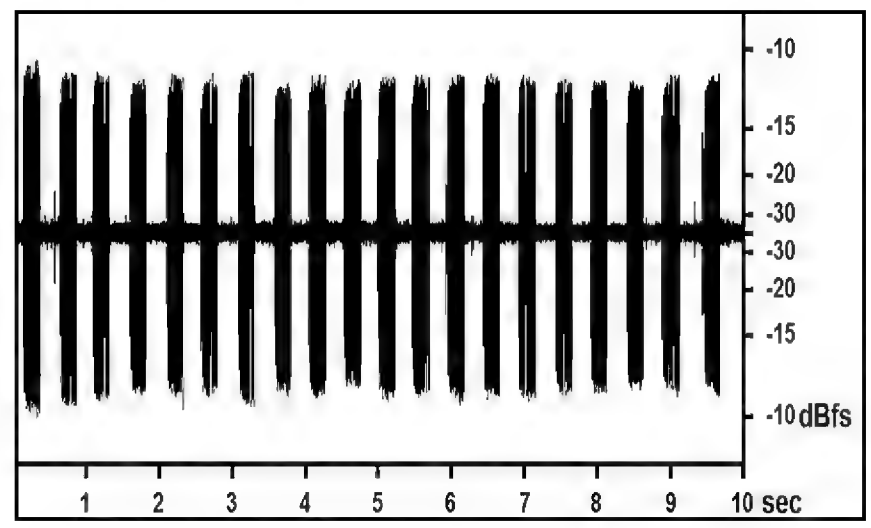

Figure 6. Oscillogram of a 96 kHz audio sample of *Natula averni*, Portixeddu, 24.IV.2018.

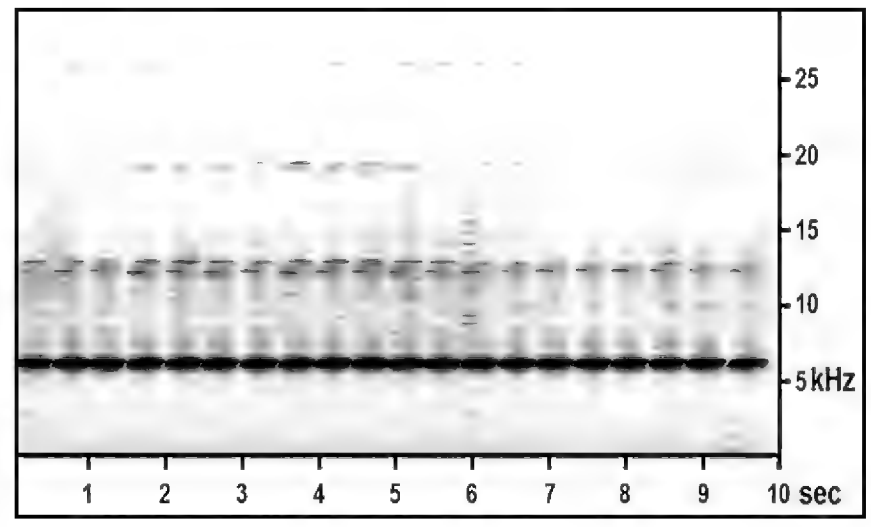

Figure 7. Time/frequency spectrogram of a 96 kHz audio sample of *Natula averni*, Portixeddu, 24.IV.2018.

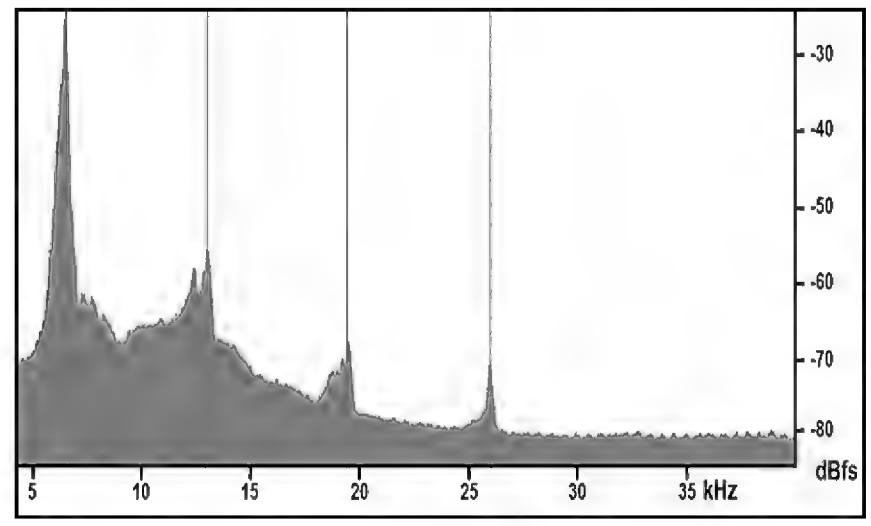

Figure 8. Frequency/volume analysis of a 96 kHz audio sample of *Natula averni*, Portixeddu, 24.IV.2018. Blue lines mark the fundamental frequency and its harmonics.

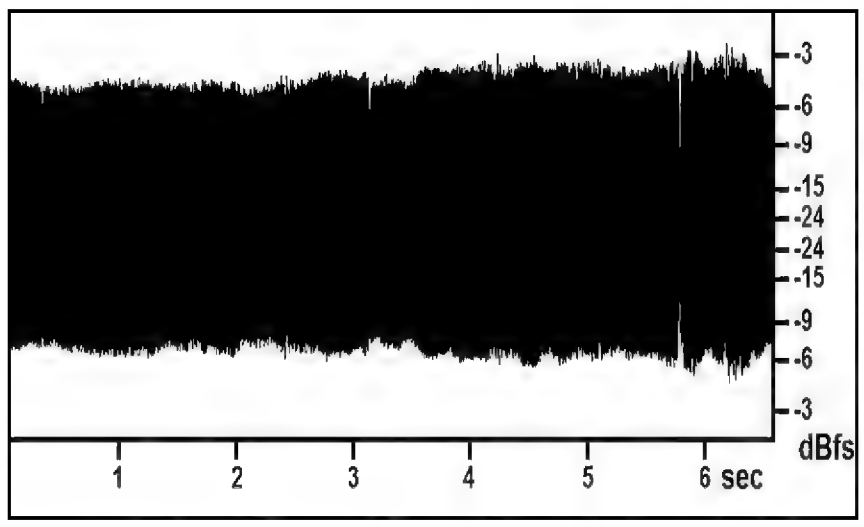

Figure 9. Oscillogram of a 96 kHz audio sample of *Brachytrupes megacephalus*, Capo Pecora, 21.IV.2018.

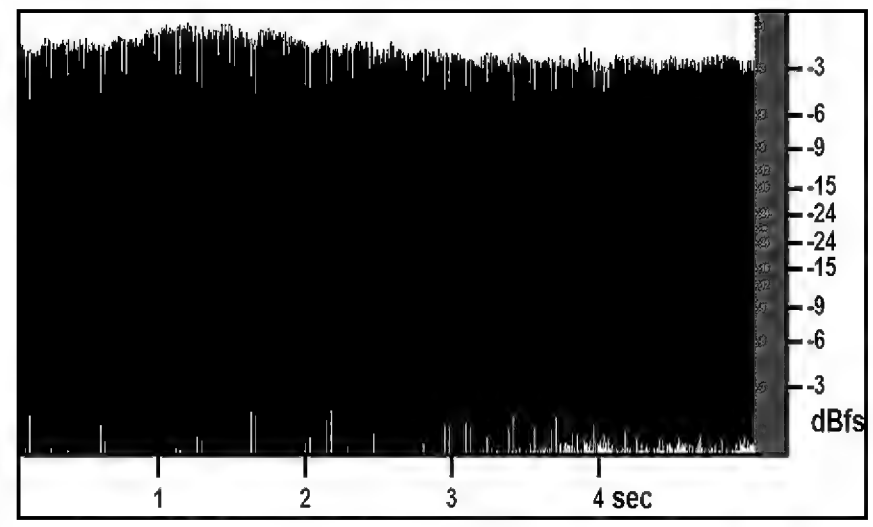

Figure 11. Oscillogram of a 250 kHz audio sample of *Brachytrupes megacephalus*, Capo Pecora, 22.IV.2018.

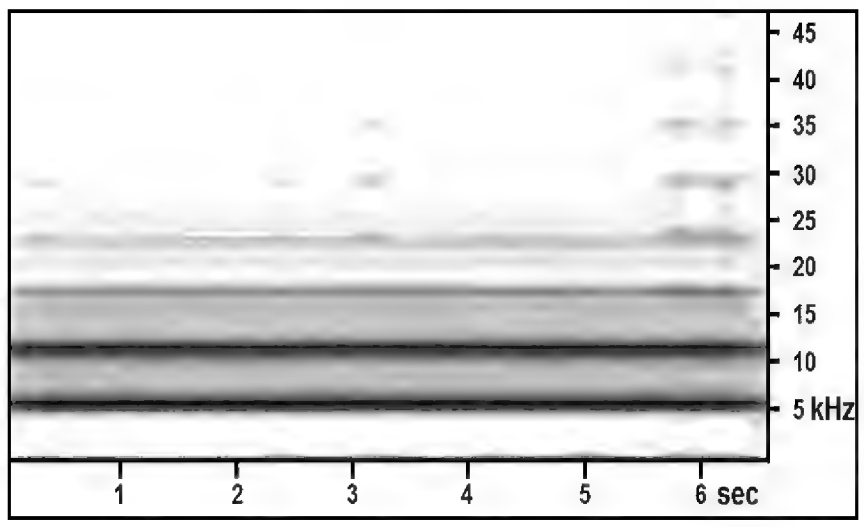

Figure 10. Time/frequency spectrogram of a 96 kHz audio sample of *Brachytrupes megacephalus*, Capo Pecora, 21.IV.2018.

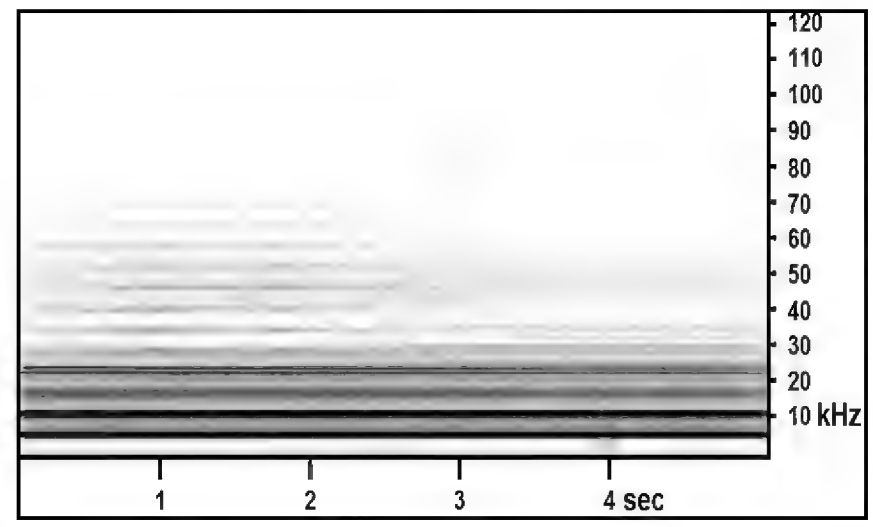

Figure 12. Time/frequency spectrogram of a 250 kHz audio sample of *Brachytrupes megacephalus*, Capo Pecora, 22.IV.2018.

All the main elements are reported in Table 3.

A strong harmonic structure is observed in the frequency/volume analysis of the wide band, 250 kHz sampling frequency recording, with a geometrical progression of octaves delimited by peaks corresponding with the fundamental frequency and its first twenty-one harmonics in geometrically decreasing intensity. All the high order (above 50 kHz) harmonics are in better accordance with a 5800 Hz fundamental, the same observed in the 96 kHz recordings. An accessory cusp can be observed in the 15–50 kHz range, around 1500 kHz below each harmonic frequency, with comparable intensity. A more complex cluster of secondary peaks, among which a two-cusps, regularly repeated pattern, is evident at middle octave in the 5–58 kHz range. Despite the technical differences in microphone sensitivity and recording device, the analysis of the 96 kHz recording reproduces quite faithfully in the 0–48 kHz range the complex pattern described above.

CONCLUSIONS

The songs of two uncommon crickets from Sardinia have been digitally recorded in the field, one of them by a low-cost USB microphone capable of generating 0 to 125 kHz monophonic recordings, including both audible and inaudible frequencies. This device, Ultramic 250, by generating results consistent with other recording methods and by providing useful information about high-frequency components above 20 kHz and up to 125 kHz, allowed to observe a previously unreported high-order harmonic structure for the song of the biggest European cricket, *B. megacephalus*. In addition, the underreported and very elusive, *N. averni*, whose systematic status may need some revision (Odé et al., 2011), was recorded and analysed in the 0–48 kHz range, revealing a well-defined progression of harmonics above its maximum volume frequency.

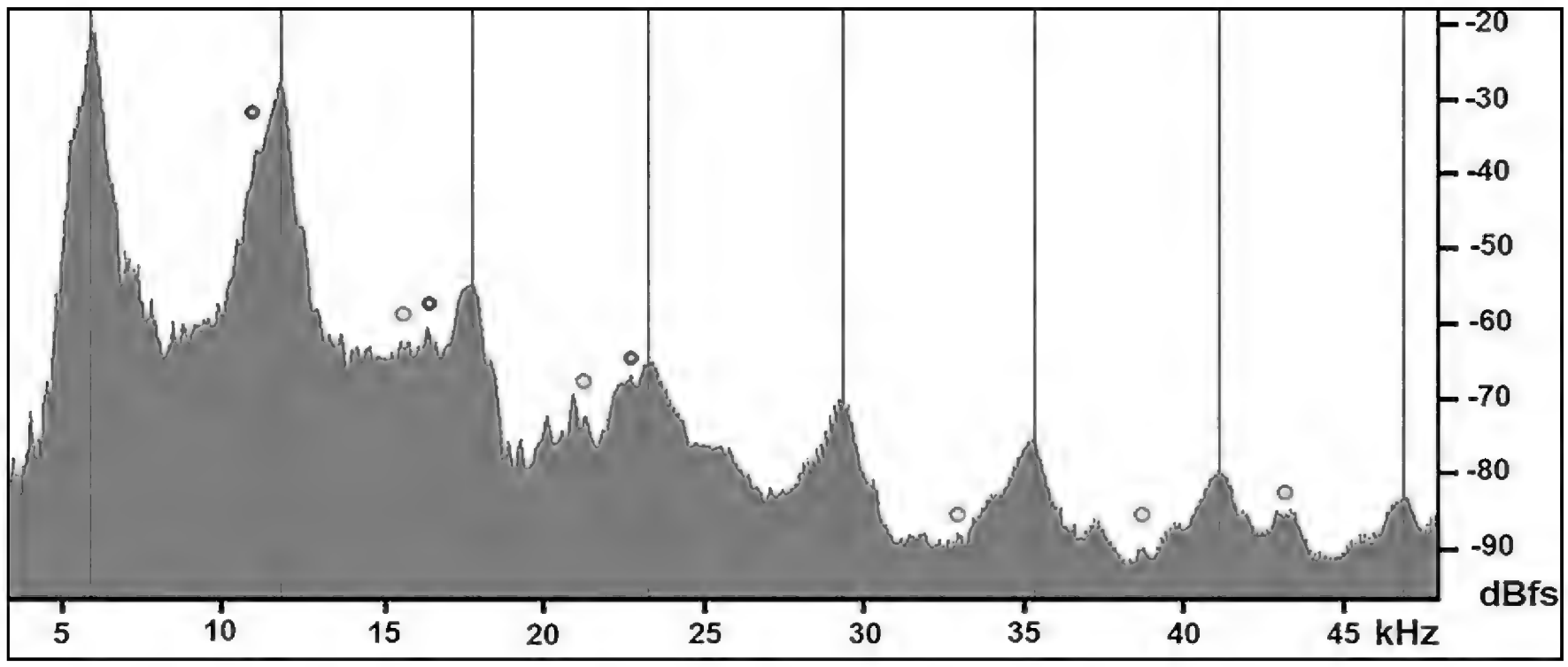

Figure 13. Frequency/volume analysis of a 96 kHz audio sample of *Brachytrupes megacephalus*, Capo Pecora, 21.IV.2018. Blue lines mark the fundamental frequency and its harmonics. Blue circles mark the secondary peaks preceding some harmonics. Magenta circles mark frequency clusters also observed in the 250 kHz recordings.

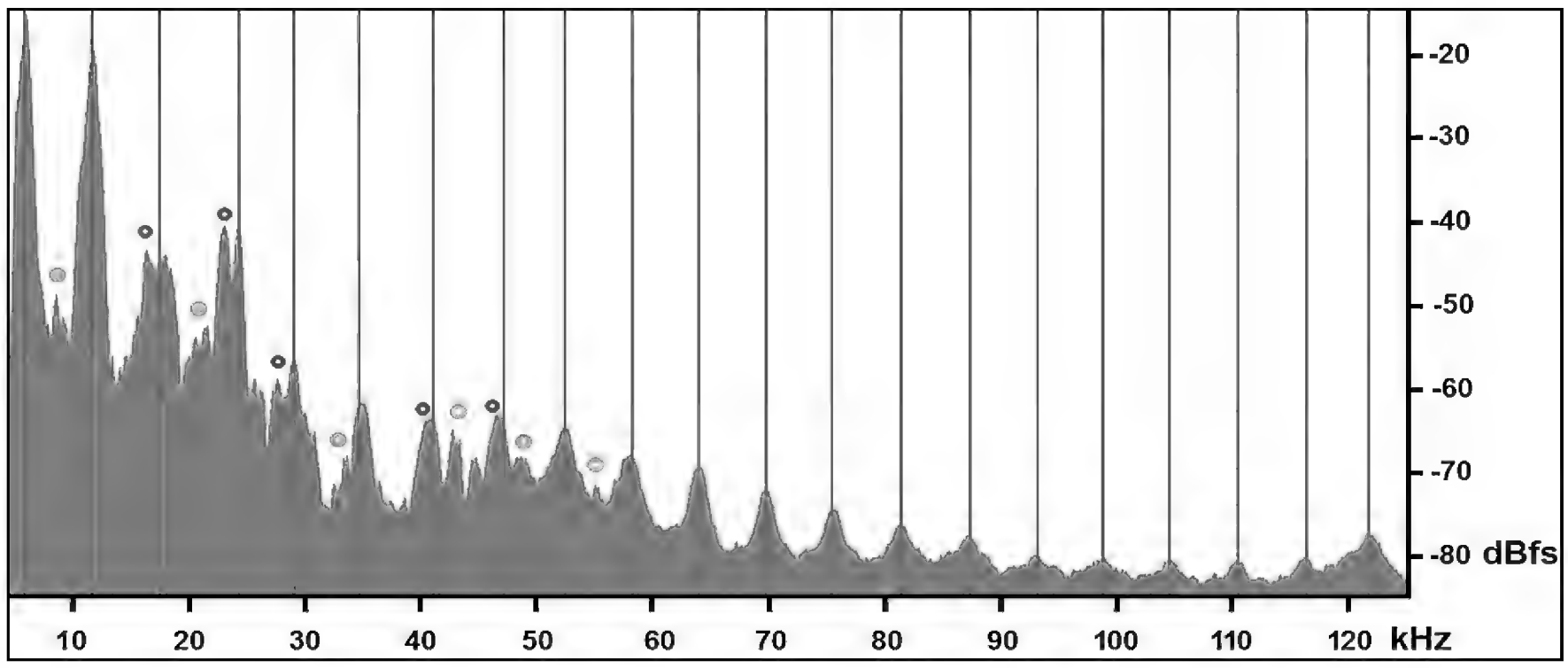

Figure 14. Frequency/volume analysis of a 250 kHz audio sample of *Brachytrupes megacephalus*, Capo Pecora, 22.IV.2018. Blue lines mark the fundamental frequency and its harmonics. Blue circles mark the secondary peaks preceding some harmonics. Magenta circles mark regularly repeated frequency clusters, some of which also observed in the 96 kHz recordings.

Considering the restricted habitat of both the species examined, the value of this report may be regarded both as ecological and biogeographical, as well as bioacoustic.

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New data on the genus *Luidia* Forbes, 1839 (Asteroidea Luidiidae) from the gulf of Oman and first record of *Luidia maculata* Müller et Troschel, 1842 in this region

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ABSTRACT As a part of the research program about echinoderms of the Gulf of Oman, *Luidia maculata* Müller et Troschel, 1842, and *L. hardwicki* Gray, 1840 (Asteroidea Luidiidae) were collected from the sandy shores of Chabahar Bay along the north part of the gulf during the period from 2015 to 2016. Here, *L. maculata* is recorded for the first time from the Gulf of Oman and Iranian waters. *Luidia hardwicki* has been previously reported from this area. Details concerning the identification and distribution range of these species are provided.

KEY WORDS Gulf of Oman; Iran; Chabahar Bay; echinoderms; *Luidia maculata*; *L. hardwicki*.

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INTRODUCTION

Luidia Forbes, 1839 (Asteroidea Luidiidae), with 50 known species and its wide distribution in the tropical and subtropical waters is the only valid genus of Luidiidae Sladen, 1889, the smallest family in the asteroidean (Clark & Mcknight, 2000; Mah & Blake, 2012; Kim et al., 2017). They live on the muddy or sandy sediments of substrates (Sloan, 1980).

Members of the Luidiidae Family are identified by having five to nine flat and straplike arms and by the presence of Superambulacral plates and paxillate abactinal surface. In the Luidiidae Family, the lateral side of the body, distinguished by just an inferomarginal plate and supero-marginals plates, are not distinguishable from the paxillae (Clark & Rowe, 1971).

Asteroidean specimens have been less studied in the gulf of Oman. To date, 6 asteroid species: *Astropecten phragmorus* Fisher, 1913, *A. polyacanthus* Müller et Troschel, 1842, *A. hemprichi* Müller et Troschel, 1842, *A. indicus* Döderlein, 1888, *Aquilonastra burtonii* Gray, 1840, and *L. hardwicki* Gray, 1840 have been reported from Iranian waters of the gulf of Oman (Khaleghi, 2010; Esfandiarpour, 2014; Panahloo, 2015).

The intertidal benthic fauna of the Iranian coasts along the Gulf of Oman were studied as a part of a research project covering the coastal waters of Jask, Chabahar Bay, and Gwatr Bay. As result, *L. maculata* and *L. hardwicki* were collected at low tide from the sandy shores of Chabahar Bay. *Luidia hardwicki* has been previously reported from the Cabahar Bay (Esfandiarpour, 2014). Because of presence of *L. maculata* in the adjacent waters of the gulf of Oman (Mortensen, 1940; Clark & Rowe, 1971; Liu, 2008; Price, 1983), observation of this species in this region was predictable. This is the first record of *L. maculata* from the Gulf of Oman and also Iranian waters.

MATERIAL AND METHODS

This study has been conducted along the Iranian waters of the Gulf of Oman. Three locations were selected as sampling sites: Qwatr Bay (25°08'N, 61°29'E), Chabahar Bay (25°19'N, 60°37'E), and Jusk (25°35'N, 58°02'E) (Figure 1). Specimens were collected from the sandy shores of Chabahr Bay at low tide by hand. Chabahar Bay is located in the north part of the Gulf of Oman along the Iranian waters. In order to keep the specimens alive after the sampling, they've been transported to the laboratory. The samples imaging has been done by digital camera and light microscope. For long time preservation, 75% ethanol has been used. Specimens were carefully examined and taxonomic studies were performed by following the identification key of James & Pearse (1969), Clark & Rowe (1971), Price (1983), and VandenSpiegel et al. (1998).

The examined materials is deposited at the Zoology Museum of Chabahar Maritime University, Iran.

RESULTS

Systematics

Classis ASTEROIDEA de Blainville, 1830
Ordo PAXILLOSIDA Perrier, 1884
Familia LUIDIIDAE Sladen, 1889
Genus *Luidia* Forbes, 1839

Ludia maculata Müller et Troschel, 1842

EXAMINED MATERIAL. Two adult specimens of *L. maculata* were collected in October 2015 and November 2016 from Chabahar Bay. 2015 specimen with seven and 2016 specimen with six arms.

DESCRIPTION. *Ludia maculata* is a large species (R/r = 7.7 to 8.1) with 6 to 7 (in this study) large, flat, and strap-like arms. Disc small and madreporite covered by paxillae. Abactinal paxillae with eight to twenty short central spinelets and about twenty-five to thirty for more slender peripheral spinelets. The paxillae that are located in mid-arm are more irregular and smaller and have fewer spinelets than the lateral ones. Large inferomarginal plates with 3 to 5 short spines and numerous spinelets. Adambulacral plate with 3 curved and flatted spines. *Ludia maculata* has a bold black mottled coloration on the dorsal surface of the body; ventral side whitish. This pattern coloration retained in the preserved specimens. There are no large bivalve pedicellariae near the mouth (Fig. 2).

DISTRIBUTION. West Indian ocean, East Africa and Madagascar, North Australia, China and South Japan (Clark & Rowe, 1971), Singapore (Vanden-Spiegel et al., 1998), Thailand (Putchakarn & Sonchaeng, 2004), Red Sea (James & Pearse, 1969), Arabian Sea (Parameswaran et al., 2017), Persian Gulf (Mortensen, 1940; Price, 1983), and Pakistan (Haque, 1969).

REMARKS. Specimens were collected from the intertidal sandy-gravel shores of Chabahar Bay. In this research, a very rare form of *L. maculata* (with 6 arms) was found in the sampling site. This is the first report of *L. maculata* from the Gulf of Oman and also Iranian waters.

Luidia hardwicki Gray, 1840

EXAMINED MATERIAL. About 20 specimens of *L. hardwicki* (all of them with 5 arms) were found in the sandy shores of Chabahar Bay at low tide (2015 to 2016, legit Yaser Fatemi).

DESCRIPTION. A medium sized asteroid species (R/r = 5.2) with moderately large, flat, and strap-like arms (five); Disc small and madreporite covered by paxillae. Preserved specimens pinkish in color; there are no marked color pattern on the dorsal surface body; abactinal paxillae with six to fifteen subequal, blunt-tipped, central spinelets, and with thirteen to twenty-three more slender peripheral ones. Mid-arm paxillae are more irregular and smaller, and have fewer spinelets than the peripheral ones. Large slender pedicellariae on the outer part of some adambulacral plates; a single slender spine and numerious smaller spines encircled the inferomarginal plates. Large bivalve pedicellariae located near the mouth (Figure 3).

DISTRIBUTION. West Indian Ocean, North Australia, China, and South Japan, Red Sea (Clark & Rowe, 1971), Singapore (VandenSpiegel et al., 1998), Thailand (Putchakarn & Sonchaeng, 2004), Arabian Sea (Parameswaran et al., 2017), Persian

Figure 1. Map of the sampling site.

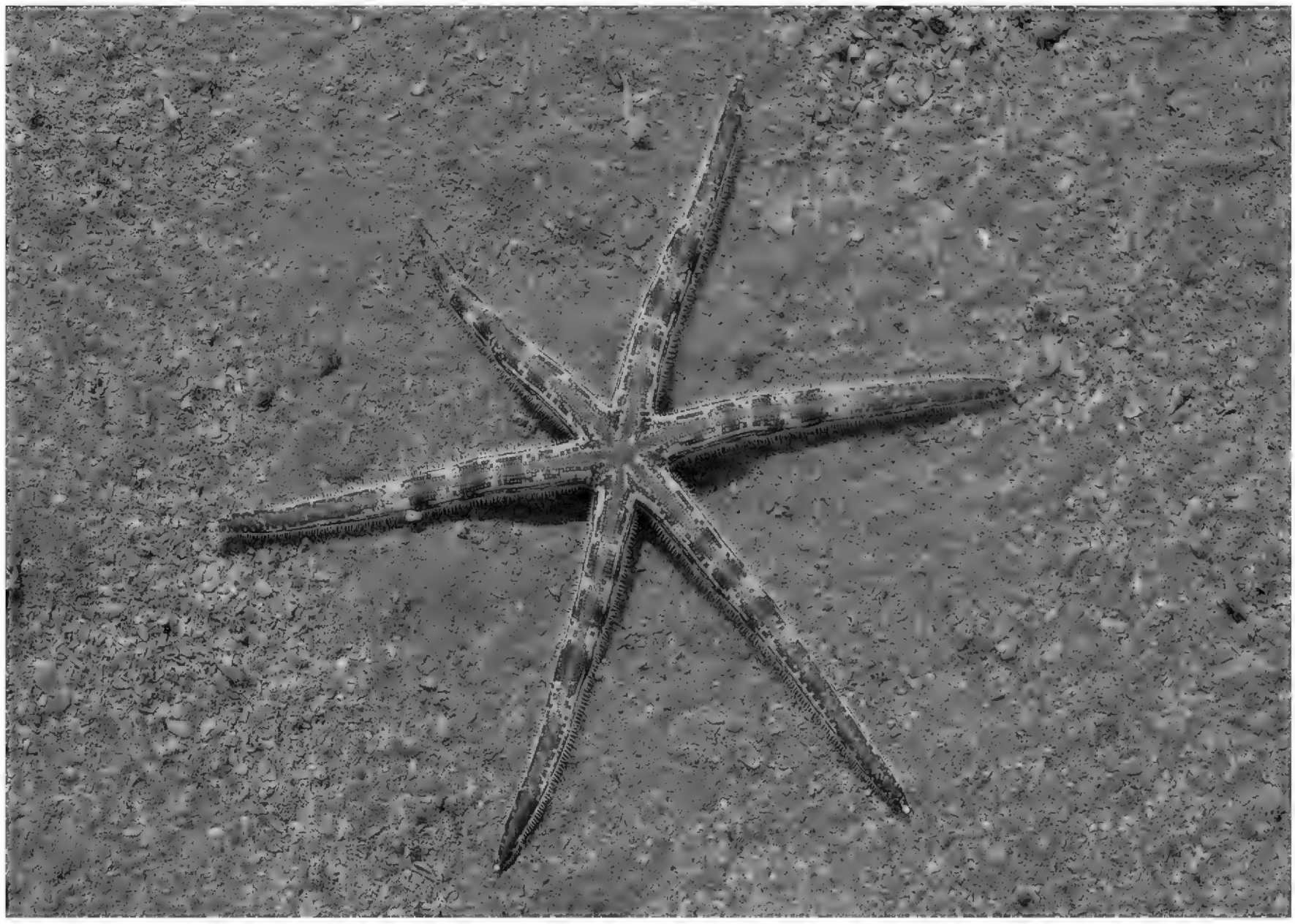

Figure 2. Living specimen of *Ludia maculata* Müller & Troschel, 1842, in the sampling site, Chabahar, Iran, Gulf of Oman. Frontal view. Photo credit: Yaser Fatemi.

Gulf (Mortensen, 1940; Price, 1981), and the Gulf of Oman (Esfandiarpour, 2014).

REMARKS. *Luidia hardwicki* has been previously reported from the gulf of Oman by Esfandiarpour (2014).

Key to the species of *Luidia* genus known from the Gulf of Oman

1. Large specimens with 6 to 7 arms (up to 9); bold black mottled coloration on the dorsal surface of the body; up to twenty central spinelets can be seen for each abactinal paxillae and up to thirty for peripheral ones; there are no large bivalved pedicellariae in the adoral margin of oral plates…..…… .....*Ludia maculata* Müller et Troschel, 1842

2. Specimens with five arms (rarely 6); without marked color pattern on the dorsal surface of the body; abactinal paxillae with six to fifteen subequal, blunt-tipped, central spinelets, and with thirteen to twenty-three more slender peripheral ones. Pinkish in color ……..…..*Luidia hardwicki* Gray, 1840

DISCUSSION AND CONCLUSIONS

Luidia maculata and L. *hardwicki* of the Luidiidae family were collected from October 2015 to November 2016 by hand in the intertidal waters of the Iranian costs of the Gulf of Oman. *Luidia maculata* is an indo-pacific species that lives in sandy sediments of substrate. It can be easily distinguished from the related species by the number of arms (usually 7 and very rarely 6) and the bold black mottled coloration of the back (Price, 1983).

Luidia maculata previously reported from the Persian Gulf and Arabian Sea (Mortensen, 1940; Haque, 1969; Clark & Rowe, 1971; Price, 1983). Therefore, the presence of this species in the gulf of Oman was predictable. Price (1983) reported *L. maculata* (7 specimens) from the subtidal sand and grass beds at 5–11 m depth of Dammam Channel and Tarut Bay (Persian Gulf). Mortensen (1940)

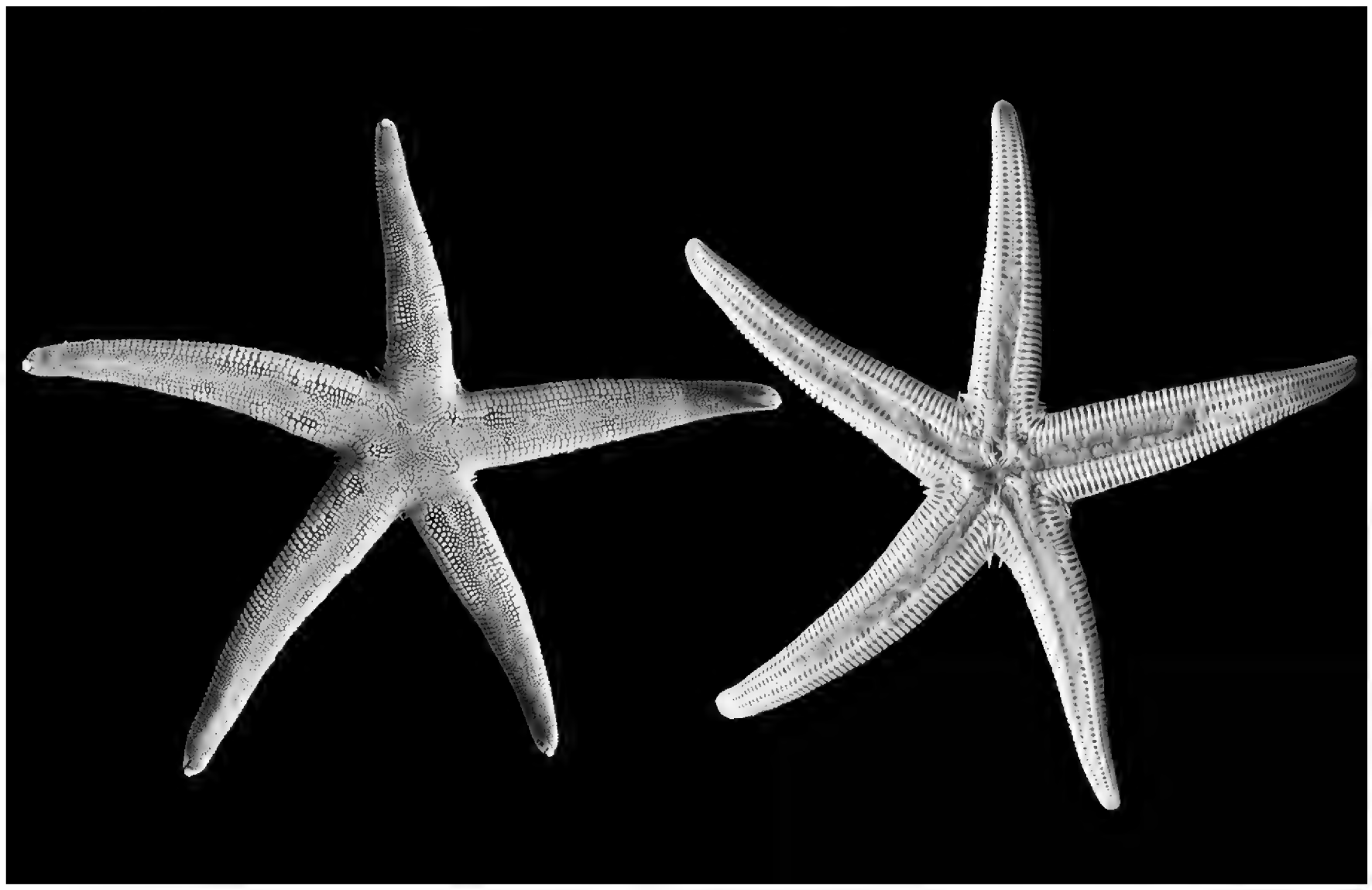

Figure 3. Preserved specimen of *Luidia hardwicki* Gray, 1840, in aboral view (left) and oral view (right). Chabahar, Iran, Gulf of Oman. Frontal view. Photo credit: Yaser Fatemi.

collected three specimens of *L. maculata* at 33 m depth of northeast of Bahrain (Persian Gulf). *Luidia maculata* was reported for the first time in Pakistan in the survey study of Haque (1969).

Luidia harwicki is a common species of Luidiidae that has been recorded from the northwest of the Indian Ocean (Mortensen, 1940; Clark & Rowe, 1971; Esfandiarpour, 2014; Parameswaran et al., 2017). It can be easily observed on the sandy shores of Chabahar Bay at low tide.

Chabahar Bay is semiclosed, with gentle slope and sandy beach that has favorable conditions for the survival of echinoderms. Both species cannot be found in the Jusk and Gwatr Bay. It can be related to the extreme waves in the coast of Jusk and to muddy sediment of mangrove forest of Gwatr Bay (Fatemi et al., 2015). Unlike the other studies, *L. maculata* was collected in the intertidal zone of Chabahar Bay at low tide. According to the literature, it can be said that *L. maculata* is a moderately rare asteroid species in the northwest part of Indian Ocean. However, *L. hardwicki* is a common asteroidae in this region.

This study represents the first record of *L. maculata* in the Gulf of Oman and Iranian waters. Cabahar Bay is the northernmost records in the Indian Ocean for the distribution range of *L. maculata*. From the family of Luidiidae, *L. prionota* Fisher, 1913, has been recorded from the Persian Gulf, Red Sea, and Arabian Sea (Mortensen, 1940; Clark & Rowe, 1971). Thus, the presence of this species in the Gulf of Oman can be predictable.

More studies are required to find the *L. prionota* and other asteroidean species of the Gulf.

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Is biodiversity aging? Heuristic questions on the taxonomic diversity in the Phanerozoic

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ABSTRACT The trend of numerosity component of diversity was analysed from palaeontological data, according to various time intervals and taxonomical ranks. Taxa numerosity of lower (with respect to family), with a nearly exponential increase, vs. higher ranks (from order to macro-taxon), with mainly a logarithmic trend, confirms to follow quite different patterns over time. This dataset seems to fit with a more assortative hypothesis, for higher taxa and a more divisive one for lower taxa. Then, a model was built to quantify the relative weight, over time, of the above hypothetical evolutionary components of various taxa ranks; such ranks were identified, in a palaeontological approach, by the maximum number of recorded taxa or, in a phyletic approach, by the time duration based on genetic data. The trends obtained by this model agree with observed records and the hypothesis, to be verified, of a quite different evolutionary origin of macro- (phylum, class, order ranks) and micro-taxa (genus and species), during the transition between two main time phases: the first (evidently more assortative), mainly linked to the lateral sharing of characters, the second (evidently more divisive), mainly influenced by the ever growing morpho-physio-genetic isolation even for the protection of complex adaptations, as in the present, "modern species".

KEY WORDS Evolutionary models; genetic timing; macroevolution; taxonomic diversity.

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"Iamque adeo fracta est aetas effetaque tellus vix animalia parva creat quae cuncta creavit saecla …" (Lucretius).

INTRODUCTION

Since evolutionary theory was accepted as the only possible rational explanation of the life diversification in the biosphere - e.g., Gould (2002); see also Mayr (1982), evolutionary processes have received many and different interpretations. Two main question points have been particularly investigated: (i) the basis of the biological variability, and (ii) the factors orienting its evolution: see, e. g., Gould (2002). Moreover, genetics has provided experimental evidence of the "transition" from a species into another species, within a framework that has been defined "microevolution".

However, there has been a contextual difficulty in explaining the great differences in morpho-physiological models, occurring among high-rank taxa like phyla, classes, and orders by means of merely micro-evolutionary processes (Stanley, 1979).

Therefore, Stanley (1979) hypothesized a "decoupling" of selective ranks, between microevolution (selection among individuals) and macroevolution (species as individual units of selection, in macro-evolution, in analogy with specimens in micro-evolution).

Hence, in order to explain the sudden appearance of organization plans totally innovative, evolutionary biologists invoked mechanisms - see Gould (2002) for an extensive synthesis, that include exaptation, polyploidization, neoteny, lateral transmission, and the passages from individual to colony (or society) to super-individual, related to patterns of complexification of genic complexes, up to the confluence of morphophysiological characters of different taxa in a single one through, e. g., endosymbiosis, parasitism, trophism, hybridization even between well distinct groups, up to the confluence of various taxa into a single biological cycle (Landman, 1991; Doolittle, 1998, 2000; Goldenfeld & Woese, 2007; Gould et al., 2008; Sanchez–Puerta & Delwiche, 2008; Archibald, 2009; Dagan & Martin, 2009; Kleine et al., 2009; Rogozin et al., 2009; Liu, 2011); see also Williamson (2003); recently, Oakley (2017), with a formal and general approach.

It remains, however, to be discussed whether the complexification of the above-mentioned mechanisms is just a sub-group of the whole complexification, which includes also the dimensional increment, coloniality, sociality, and even the indirect development (with or without metamorphosis).

In addition, it has been observed that the genetic roots of micro- and macro-evolutionary aspects may be surprisingly coincident (Gompel et al., 2005).

According to a classical view: e.g., Rensch (1959), the emerging of new, increasingly differentiated taxa into the history of the life on earth, should have been gradual in time (i.e. from species to genus, from genus to family, from family to order, etc). However, since the Palaeozoic, there has been a tumultuous appearance of the majority of the greater taxa, whereas in more recent times there has been a huge increment in the appearance of the number of minor taxa such as species and genera (Signor, 1978, 1982, 1985; Raup, 1983; Benton, 1993, 2001; Smith, 2001; Sepkoski, 2002; Lane & Benton, 2003; Bush & Bambach, 2004; Jackson & Johnson, 2001; Holland & Sclafani, 2015; but see, e.g., Mc Gowan & Smith, 2008; Ruban, 2010). Nearly all these minor taxa can, however, be inserted into already-known greater taxa (orders, classes, and phyla).

The family rank, appears to be intermediate and pivotal even in relation to the previous topic and the relevant trend (Sepkoski, 1979; Benton, 2001; Benton & Emerson, 2007) appears as intermediate between that of orders and genera, perhaps due also to the variety of taxonomic approaches.

There was also a great difference among the various models proposed to interpret the patterns of taxonomic diversity in the Phanerozoic (see, e.g., Valentine, 1970; Valentine & Moores, 1970; Schopf, 1979; Wise & Schopf, 1981; *etc.*). This great difference can be interpreted as a possible influence of the taxonomic rank chosen by the different authors (Lane & Benton, 2003; Markov & Korotaiev, 2007), i.e. a low rank (species-genus) (Raup, 1976a, b; Bambach, 1977; Valentine et al., 1978) or at high-rank (order etc.) (Gould et al., 1977; Sepkoski, 1978). Indeed, also the "consensus" model (Sepkoski, 1978) showed that there was a high numerosity of higher taxa in the most ancient phase of the history of life, and a higher numerosity of the lower taxa in the most recent phases. In particular, both the low taxa numerosity during the early phases of the history of life, and the Cenozoic explosion of low-rank taxa, cannot be attributed to artefacts (Signor, 1978, 1982, 1985). Anyway, Benton & Emerson (2007) did not consider that it is possible to identify a single model explaining the whole variety of contexts and taxonomic ranks. Important new discoveries (such as for the "evo-devo") have strongly challenged and re-evaluated old certainties (Gould et al., 1977; Gould, 1989, 2002). For instance, on the homologies and analogies of characters, the prevalent stability of evolution over time with relatively strong accelerations through cladogenesis, hierarchic selection models (from gene to species and beyond), evolution by reductive instead of addictive diversification of the gene complexes, and historical evolution of a same aptitude-to-evolve and the role of the complexity science in the evolutionism (Kauffman, 1993).

This fact has carried to important intuitions, although these intuitions have not been always demonstrated: e.g., the structural approach to the form-function relationship (i.e., exaptation) or a distinct "historic" determinism of the macroevolution, partly differing from that of microevolution (Gould, 2002).

So, it has also been proposed to place, together with the classical view of an adaptive evolution through qualitative mutations, also an evolution through progressive complexification due to the increasing of the hereditary pool (Ohno, 1970; Bateson, 1979; Taylor, 1979; Omodeo, 1985, 2010). In

the present paper, the expression "*assortative evolution*" was preferred, as evolutionary complexification linked to the confluence of distinct and different taxonomic sources.

Palaeontology is ever fundamental to understand the distribution and evolution of biological taxa during the history of the Earth and to define the time intervals characterizing the main phases of evolution. Today, such studies are positively supported by neontological advances, e. g., of genetics and its molecular bases. In any case, there is now a wide consensus, e.g., in determining as the beginning of the Phanerozoic the formation of a "modern" atmosphere, conditioned by O2, around 600 Ma (Farquhar, 2009; Omodeo, 2010; Karhu, 2012). Possibly, in time, the biosphere, which was nearly virtual in the early phases, assumed a real volume becoming with time more and more influenced, even in its abiotic components, by living organisms which were increasingly conditioned in their morphological evolution by bio-coenotic interactions and the relative adaptive responses (Butterfield, 2007).

Hence, if the early interactions occurred mainly with the abiotic environment (i.e. they could be fundamentally biochemical interactions), the successive interactions were mainly done with the biotic environment, through ecological niche processes such as competition, predation, and mediating characteristics such as vagility, modularity, body size increases, etc. Hence, with the Phanerozoic, it became possible the reliable identification of the greater phyletic groups based on macro-morphology of taxa.

Indeed, until now the determinism of the early phyletic diversification versus the later diversification of the lower taxa remain extremely doubtful.

A suite of analyses was applied to these morphologically identified taxonomic groups, in order to test whether the numerosity pattern over time of the high-rank taxa (phyla, classes, orders; see, e. g., Contoli & Pignatti (2011) and of the low-rank taxa (genus, species) may have a substantially different causal origin; so perhaps extending the ideas of Woese (2002; 2004) up to the last part of the pre-Cambrian.

In particular, it has been verified:

whether and how much the trends of numerosity of more or less inclusive taxonomic ranks (e. g.: orders v.s genera) differ from each other over time;

what are the implications, about these trends, of the theoretical assortative or divisive outlines;

if these patterns prevail in the outlines observed in real taxonomic ranks;

the predictivity of a model based only on the above mentioned theoretical outlines, in relation to the relative numerosity of taxonomic ranks according to the fossil record.

MATERIAL AND METHODS

Sources of data

To analyze the curve fitting of observed numerosity of orders and genera over time, the data reported have been used, even in a graphic way, by Benton (2001).

The macrotaxa (see above in the text) and orders were considered "large taxa", "small taxa" the genera and species. If one compares between them a rank of a small taxa with one of the large taxa without emphasizing most of the differences, among the possible comparisons in the hierarchical succession of ranks, orders and genera are the least distant and, therefore, their comparison is the most prudent.

Chronology

To evaluate the temporal patterns of the record, considering also the observations of Benson & Mannion (2011) and Lloyd (2011), the time values of the geological periods were not used directly; indeed, in order to estimate the numerosity of taxa, the time of passage from increasing and decreasing trends or *vice versa* has been used, i. e., the maxima and minima peaks separating following trends.

When it was convenient, the record was subdivided into intervals of 100 million years, utilizing the highest number of taxa observed, independently from conventional boundaries of geological periods, in order to buffer the more or less random environmental fluctuations on which the above boundaries are usually based.

Indeed, considering the maximum values for each period means taking into account the real potential for each period, thus avoiding all those contingences which may have conditioned stochastically the duration and the strength of the observed "minima" or of the "extinction crises".

For numerosity frequencies relative to families the values suggested by Raup (1976a, b) and followed by Signor (1978, 1982, 1985), Benton (2001), Jackson & Johnson (2001), Sepkoski (2002), Benton & Emerson, 2007), Smith (2007) and Contoli & Pignatti (2011), were compared.

Methodological problems about Taxonomy

The most complete dataset in terms of both continuity and regularity regards marine animals.

Owing to the obviously very different sampling completeness of neontologic vs. palaeontologic sampling, only the latter data were used, even in case of the Cenozoic.

It is also obvious that the same taxa ranks in different phyla may not be always perfectly equivalent; however, they are equivalent in terms of their relative inclusiveness and morphological differentiation relatively to the other ranks. Even the evolutionary similarity inferred by time duration data based on genetic distance (see Appendix 1 and 2, online) shows that the mean duration of taxa for each taxonomic rank should be different and distinguishable, even if partially overlapping.

So, in the model, instead of the absolute ones, it has been preferred to use frequencies, relatives to that of families, as a pivotal taxonomic rank.

To test, with the model, the hypothesis that the trends over time of different taxonomic ranks depend on their relative numerosity with respect to the intermediate one of the families, values from literature were needed, even if approximate.

Analysing the whole palaeontological record, Sepkoski (2002) seems to suggest that there is one-order of magnitude difference among the numerosities of successive taxonomic ranks.

Other authors (Jackson & Johnson, 2001) consider that the ratio between coexisting genera and species should be between 1 to 10 and 1 to 5.

Benton (2001) inferred a ratio of 1 to 4 between families and genera or of 5 to 1 between families and orders.

Moreover, according to Smith (2007; Fig. 7), the average ratio between species and families is ≈64; so, it was inferred, between genera and families, the average ratio of 8 to 1 $\approx\sqrt{64}$.

Lastly, the ratio of ≈0.034 between macrotaxa and families is implicitly suggested by Contoli & Pignatti (2011).

Analogically, between orders and families, it was inferred the average ratio of 1 to 5 $\approx\sqrt{0.034}$.

Therefore, according to the above mentioned Authors, the minimum and maximum frequencies, related to families, were, for macrotaxa, respectively ≈0.01 and ≈0.035 (rounded up to 0.04); for orders ≈ 0.1 and ≈0.2; for genera ≈4 and ≈10; for species ≈20 and ≈100.

It is clear that, for this last rank, a homogeneous concept of species would be needed, for the study of the origin and end of the same, taking also into account, the appropriate observations of Ezard et al. (2011).

Then, the model was tested with the above 2 series of relative frequencies.

Biodiversity

To be emphasized, the present work was not looking for a richness analysis, but for a numerosity one, thus not linked to any weighting procedure.

On the other hand, numerosity can be considered, not only as a weighting base (e.g., for richness evaluation; see also Ganis, 1991; Contoli Amante, 2007, Contoli Amante & Luiselli, 2015), but also in itself, especially if pertaining to the same scale of data source.

In order to make a proper taxonomic numerosity analysis at the biospheric scale, it is necessary to apply an approach not influenced by evenness.

So, the unweighted taxa numerosity data from literature was directly used.

The model

To propose an explicative model of different trends observed in various taxa ranks, it was needed to parameterize their characters. Note that, if the biological meaning of the various taxa ranks is strongly subjective, nevertheless the above ranks, by definition, represent a hierarchic series of taxonomic ranks (subdividing the same set of organismic individuals) from the less to the more comprehensive and, so, including, among their individuals, respectively a greater or a lesser "evolutionary similarity".

Obviously, in a given lineage, a taxonomic rank is more comprehensive, while its numerosity is less, i. e. the numerosity of the taxa of the same rank; and *vice versa*.

So, for each taxonomic rank, the average evolutionary affinity between the various taxa can be expressed by the numerosity of the given taxonomical rank. Indeed, since the main taxonomic ranks are formally the same, across the whole life in the earth, every taxonomic rank includes and subdivides the whole fossil record and its diversification range; so, if that range is subdivided on more v.s fewer single taxa of a given rank, each of the above, single taxa must be, conversely, fewer v.s more different in its evolution; it results that higher numerosity at a given rank corresponds to higher average evolutionary similarity in a given taxon of the same rank.

The work was interested to compare between taxonomic ranks, not their absolute values; thus, the relative numerosity, with respect to families, of taxa of each rank will be the searched parameter of the modeling analysis.

The model can be applied to any temporal scale starting with the Phanerozoic and ending with the present.

Palaeontology

The maximum recorded number of taxa pertaining to the "i" rank can help to parametrize the various ranks. Moreover, the taxa numerosity of "i" should be directly proportional to an "evolutionary similarity" within such a taxon rank.

In the hypothesis to be modelled, in a first phase (assortative and logarithmic), the evolutionary divergence was mainly expressed by more comprehensive and, so, less rich taxa ranks; in a following phase (divisive and exponential), by less comprehensive and richer taxa.

In the "taxa in time" model, a logarithmic and an exponential component inversely linked to maximum number of taxa were summed, among the recorded time periods of the Phanerozoic.

By dividing the "i" numbers of taxa (pertaining to the taxonomic ranks of macrotaxa, orders, families, genera, and species) by that of families, as an intermediate taxonomic rank, it has been obtained:

N° taxa "i" originated at "t" time =
= k [log (t/t "final" x taxa "families"/taxa "i") +
+ exp (t/t "final" x taxa "i"/taxa "families")].

Genetic data

Methods were, since long time, conceived and refined, even when with not ever converging results, to evaluate the splitting time among taxa, estimating so also their duration. Altogether, clear differences can be observed among the various taxonomical ranks: in general, the more the duration of a taxonomic rank, the more it was comprehensive.

Their average duration in geological time can be a way to parameterize them and, conversely, to estimate their "evolutionary similarity", for the purpose of this present work, even while being aware of the partial uncertainty of these estimates (cfr. e. g. Warnock et al., 2011; Duchène et al., 2017).

In this perspective, from various sources (Novacek, 1992; Michaux et al., 2001; Dubey & Shine, 2010; Hedges & Kumar, 2009; Murphy et al., 2012), it has been inferred time duration of a lot of taxa examined by the above Authors and pertaining to the above 5 ranks; then, an estimation of minimal, maximal, and average duration of each taxonomic rank was obtained.

In order to prevent that the average mean of "i" could be influenced too much by:

- the numbers of richer or poorer taxa;
- the maxima or minima, perhaps mainly subject to errors as outliers, were excluded, from utilized data, the extreme maxima and minima;
- were averaged the minus- and plus-variant data of the same level (i. e., first and last, second and penultimate, third and last-but-two, etc.), up to means regularly invariant in the first significant numbers.

As for palaeontological data, for genetic ones it was obtained:

N° taxa "i" originated at "t" time =
= k [log (t / t "final" x duration of taxa "i"/duration of "families") +
+ exp (t / t "final" x duration of "families"/duration of taxa "i")].

Relationships between palaeontological and genetic data

As yet suggested, evolutionary similarity of "i" can be conceived as directly proportional to the maximum taxa numerosity and inversely to its time duration; so, between taxa numerosity and time duration an inverse relation was expected and observed.

Extinction

From the number of taxa of "i" rank and from

their durations, considering the extinction inversely proportional to minimum duration reported to the average one, the extinction rate was estimated:
N° taxa "i" extinct at "t" time = 2 x t/t "final" time x taxa "i" at "t" time/(minimum duration of "i"/ mean duration of "i").

An algorithm proposed for the model:

N° of "i" taxa present at "t" time = N° of "i" taxa originated in "t" time - N° of "i" taxa extinct in "t" time.

i. e.,

N° of "i" taxa presents at "t" time =
= k {log [(t time / t "final" time)/mean evolutionary similarity of taxa "i" + 1] + exp (t time/ "final" time x mean evolutionary similarity of "i" taxa) -
- 2 x t time/ "final" time x "i" taxa originated at "t" time/(minimum duration of "i"/mean duration of "i")]}.

Fitting of the data

To fit the data from literature with a suitable polynomial, was searched for functions of the smaller degree compatible with a substantially maximal significance; so, all polynomials of growing degree (1, 2, 3…) were calculated and then the R2 values were regressed vs. the growing degree of the polynomials; when the plateau was attained, the polynomial of the corresponding degree was adopted (see, e. g., Figs. 4, 6).

RESULTS

Theoretical advantages and disadvantages of assortative phyla versus time and evolution

Increases in complexity are linked to the evolution, with number of adaptations increasing in relation to increasing physiological functions, body sizes, and lifespan.

Hence, an eventual lateral confluence of genes and their morphophysiological expression of characters through their genic basis may originate a new taxon, but this may be true only if the joining genomes are compatible for some essential preexisting adaptations. In the extreme hypothesis of a single new adaptation to be conserved, there will be a 75% of useful assortments (i.e., with at least one + for each character) (Table 1), whereas in the case of two adaptations, the vital crosses will be approximately 56% (Table 2), and so on. For the case of one new character added to a previous set, see figures 1, 2.

So, there should be a logarithmic decrease in the evolutionary prospective of the potential intertaxa hybrids during the evolutionary complexification.

Moreover, the adaptive advantage of a hybrid taxon should depend also on the available niche space, which tends to diminish with time due to the increasing competition strength, thus compensating *ad abundantiam* the free niche space after catastrophic events. For both reasons, during the evolutionary complexification there must be a reduction of the assortative evolution opportunities.

Recorded numerosity of taxa through time

The recorded variations in the number of lower taxa (genera) and higher taxa (orders) over time are presented in figures 3, 5.

In general, a trinomial pattern was observed, with two inflexions of importance variable from rank to rank. The two curves were very different: the initial portions of the two curves were signifi-

	TAXON "A"		
TAXON "B"	Vital character	Present	Absent
	Present	+ +	+ -
	Absent	- +	- -

Table 1. Viability of potential assortments of 1 character for each taxon.

	TAXON "A"				
TAXON "B"	Vital char. 1,2	1+; 2+	1+; 2-	1-; 2+	1-; 2-
	1+; 2+	1+ +; 2+ +	1+ +; 2+ -	1+ -; 2+ +	1+ -; 2+ -
	1+; 2-	1+ +; 2+ -	1+ +; 2- -	1+ -; 2+ -	1+ -; 2- -
	1-; 2+	1+ -; 2+ +	1+ -; 2+ -	1- -; 2+ +	1- -; 2+ -
	1-; 2-	1+ -; 2+ -	1+ -; 2- -	1- -; 2+ -;	1- -; 2- -

Table 2. Viability of potential assortments of 2 characters for each taxon.

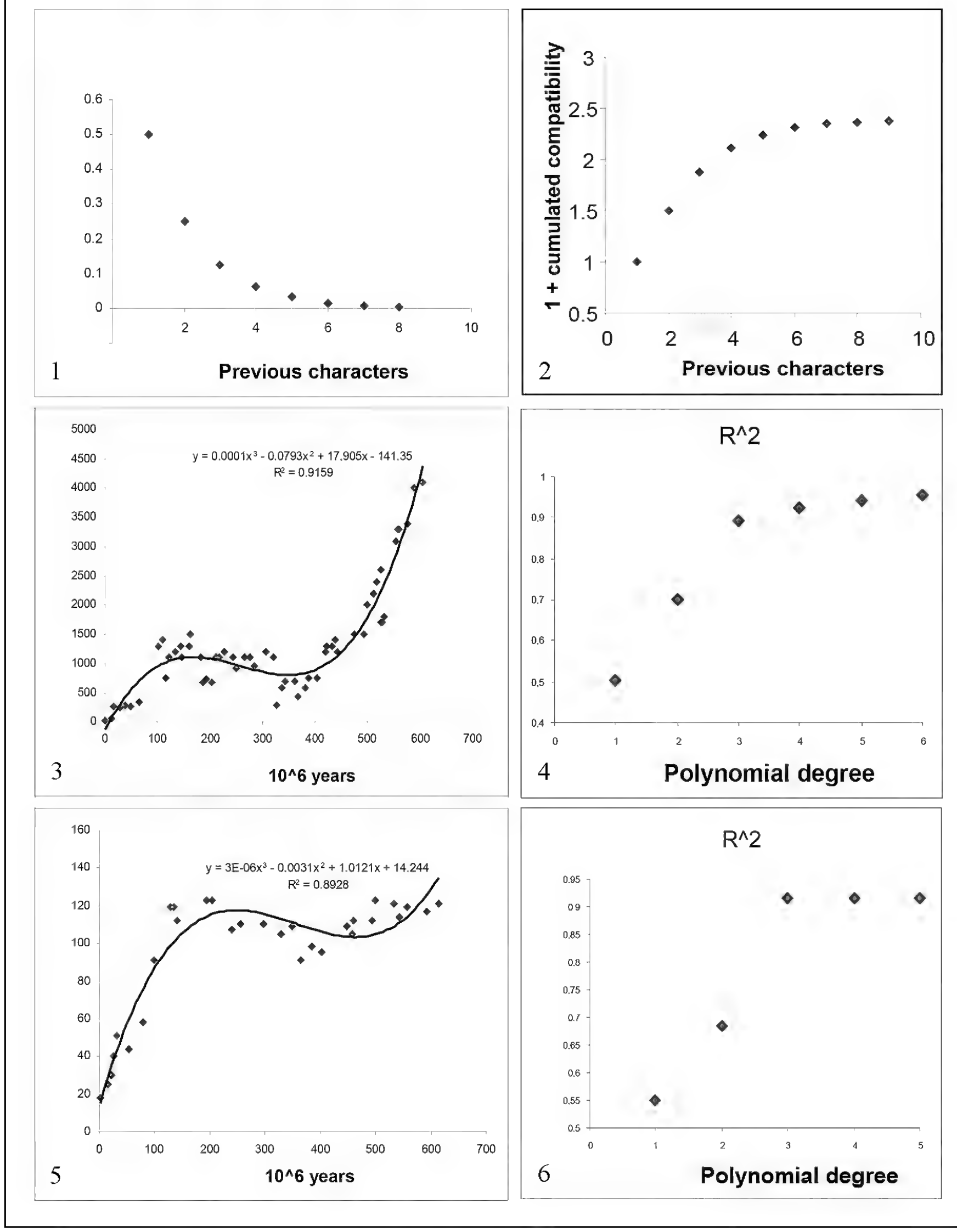


Figure 1. Theoretical compatibility of an added character to a previous set. Figure 2. Compatible assortments of an added character vs. the previous set. Figures 3, 4. Variation in the number of lower taxa (= genera) over time (Fig. 3) ; the degree of the polynomial corresponds to the flex of the "P^/degree" curve (Fig. 4). Data reported by Benton (2001). Figures 5, 6. Variation in the number of higher taxa (= orders) over time (Fig. 5); the degree of the polynomial corresponds to the flex of the "P^/degree" curve (Fig. 6). Data reported by Benton (2001).

cantly positively correlated ($P < 0.05$; one-way ANCOVA), whereas there was no correlation as for the final portions of the curves (one-way ANCOVA; $P > 0.25$).

If the significantly fitted polynomials were to be considered, the derivate is negative in the early phases and positive in the late phases. The first phases tend to prevail in the generation of the higher taxonomic categories, and the late phases in the generation of the lower categories, which tend to hegemonize the taxonomic numerosity with time elapsing.

Does time theoretically explain the passage from micro- to macro-evolution?

The theoretical relationships between time and number of taxa, for both lower taxa (species) and higher taxa (orders) (see Table 3 for the raw data), in the hypothesis of a constant time interval passing between each hierarchic passage from a given taxonomic category to another, are presented in figures 7, 8. It should be noted that the patterns seems consistent with the real data in the case of species, but not as for the macro-taxa, as this hypothetic latter pattern was even more exponential than that relative to species. Hence, in this case, the time cannot simply explain the observed patterns and, namely, the hypothesis of an "autocatalytic" increase in time of the more comprehensive taxa is untenable.

Modeling analysis

Genetic data are summarized in Table 4. Available palaeontological (Max n.r of observed taxa) and genetic (Minimum, average, Maximum duration in time) data suggest (Table 5) the relevant values of evolutionary relatedness among taxa of the same rank, relatively to the families.

In both kind of data, the model (Figs. 9–21; P: palaeontology; G: genetics) shows a trend from a more logarithmic pattern (macrotaxa and orders) to an exponential one (genera and species).

Because the model is relativized to families, values and graphs of such taxa express only the time and cannot account for any fluctuation.

The analysis of Correspondence (detrended or not), Principal components, and Clusters (according to Ward, Euclidean Distance and Single Linkage) shows the central position of time and two distinct groups of variables (both from the record and the model), representing, the first the smaller taxa ranks, the second the larger ones, well separated by the first axis representing the very bulk of variance (Figs. 22, 23); the recorded families seem to join better the smaller taxa, near to the recorded genera; nevertheless, the best fit for families (Fig. 24) is with a first degree (straight) function of time, more than a logarithmic (prevailing at first) or an exponential one (prevailing at the end), in agreement with its intermediate and pivotal position among the taxonomic ranks, confirming Hoffman (1985).

TIME	Species	Genera	Families	Orders	Macrotaxa
1	1	1	1	1	1
2	2	1	1	1	1
3	4	2	1	1	1
4	8	4	2	1	1
5	16	8	4	2	1
6	32	16	8	4	2
7	64	32	16	8	4

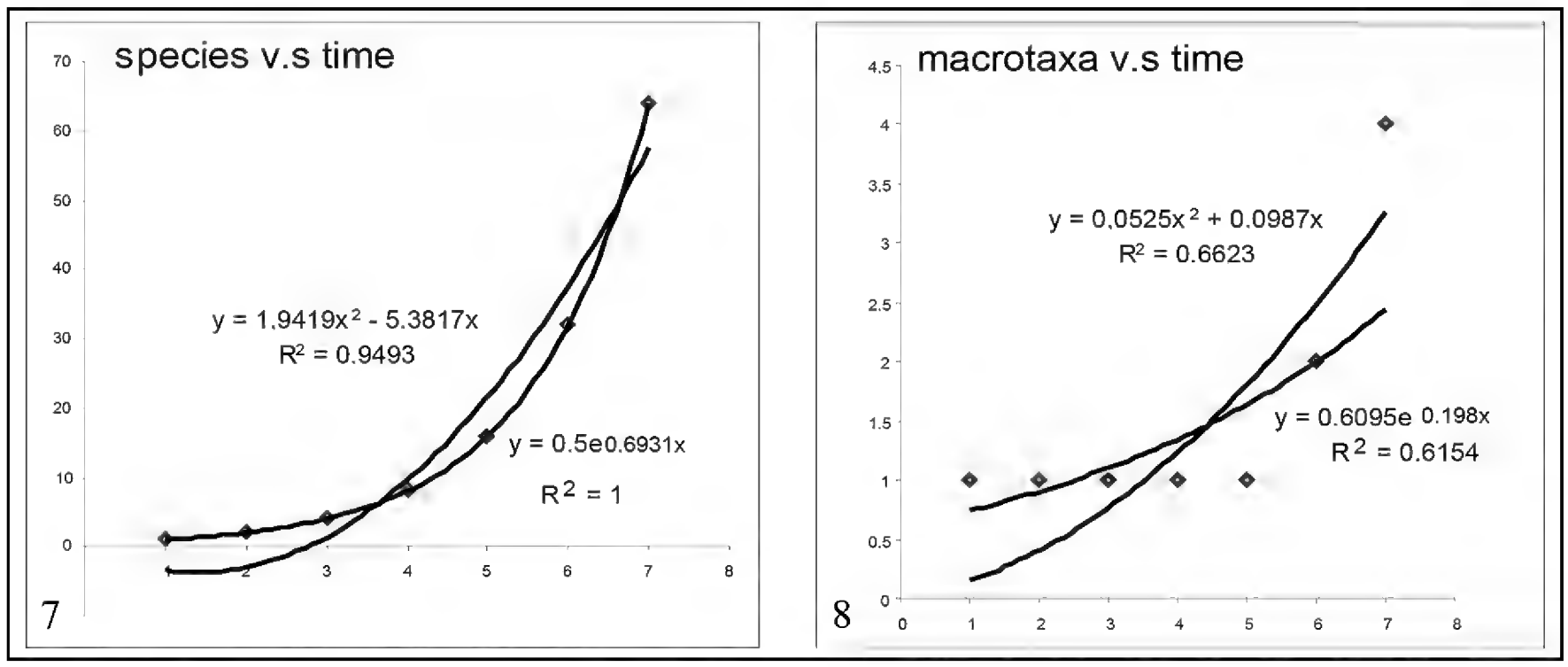
Table 3. Hypothetical hierarchical increases of various taxonomical ranks in time, according to the gradualist hypothesis.

Duration in 10^6 years	M.txa	Orders	Families	Genera	Species
Minimum	(195);208; 226;239	(32);48;50; 52	(7);10;11; 13	(5);6;7;7	.1,.2,.2,.2
Maximum	(1237);1237; 1237;1156	(470);407; 390;379	(482);308; 277;265	(113);88; 88;88	22,16,12, 11
means (m+M)/2	$[10^2]$ x [(7); 7; 7; 7]	$[10^2]$ x [(3); 2; 2; 2]	$[10^2]$ x [(2); 1; 1; 1]	$[10^1]$ x [(6); 5; 5; 5]	(11);8; 6;6
min/means; first signif. Number	.3	.3	.1	.1	.03

Table 4. Genetic duration (10^6 years).

	Macrotaxa	Orders	Families	Genera	species
PALAEONTOLOGICAL	0.01 to 0.04	0.1 to 0.2	1	4 to 10	20 to 100
GENETICAL	0.1	0.5	1	2	17

Table 5. Ratio between number of taxa at a given taxonomic rank and number of taxa at family rank, as estimator of the evolutionary relatedness.



Figures 7, 8. Relationships between time and number of taxa, for both lower taxa (species: A) and higher taxa (macrotaxa: B), in the gradualist hypothesis of a constant time interval passing between each hierarchic passage from a given taxonomic category to another (see Table 3). Fig. 7: species (ordinatae) v.s time (abscyssae). Fig. 8: macrotaxa (ordinatae) v.s time (abscyssae).

Among each of the above two very separated set of variables, the proximity between recorded and model ones of the same rank is less evident, perhaps due to the role of stochastic paleo-geologic events, clearly not affecting the model.

DISCUSSION

Synthesising the main results

In general, the patterns about the taxa numerosity which have been highlighted in previous studies (at least since Signor (1985) until Foote (2010) were confirmed, although there were some differences which were mainly due to different authors' perspectives.

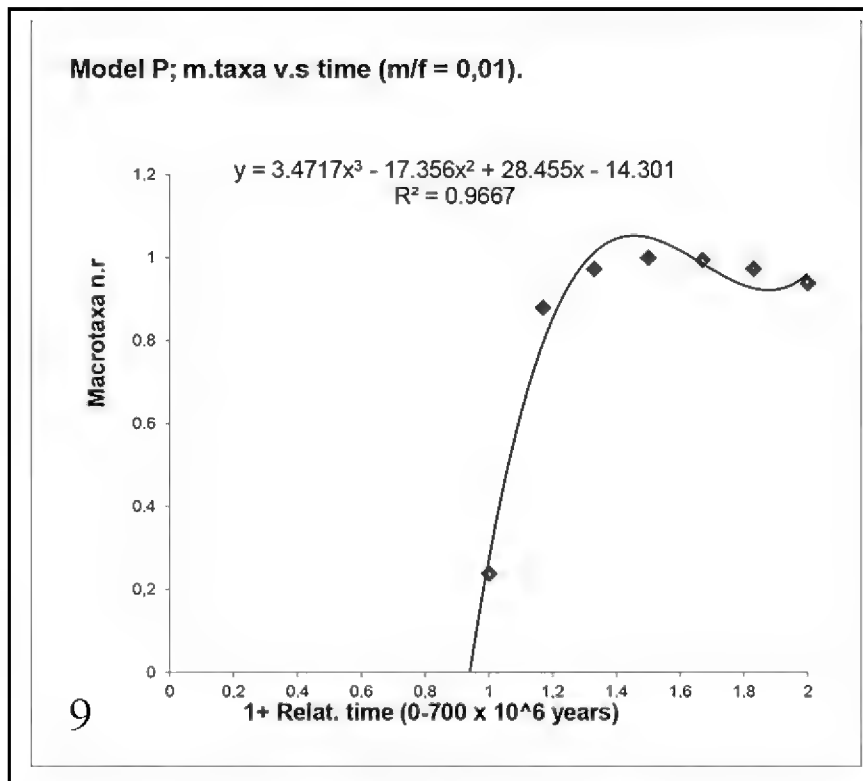
The approach developed by Alroy (2008) and Alroy et al. (2010) was not used in the present study. Indeed, although this approach (anyway, still in progress; see Marshall, 2010) is of great relevance for the standardization and comparison of the coenotic data under an ecosystemic key, it may sacrifice a conspicuous part of the available samples that are instead essential for the present study. In addition, the above-mentioned approach obviously enhances the contingent short-term oscillations and depresses the long-term oscillations, that are instead crucial for the scopes of this paper.

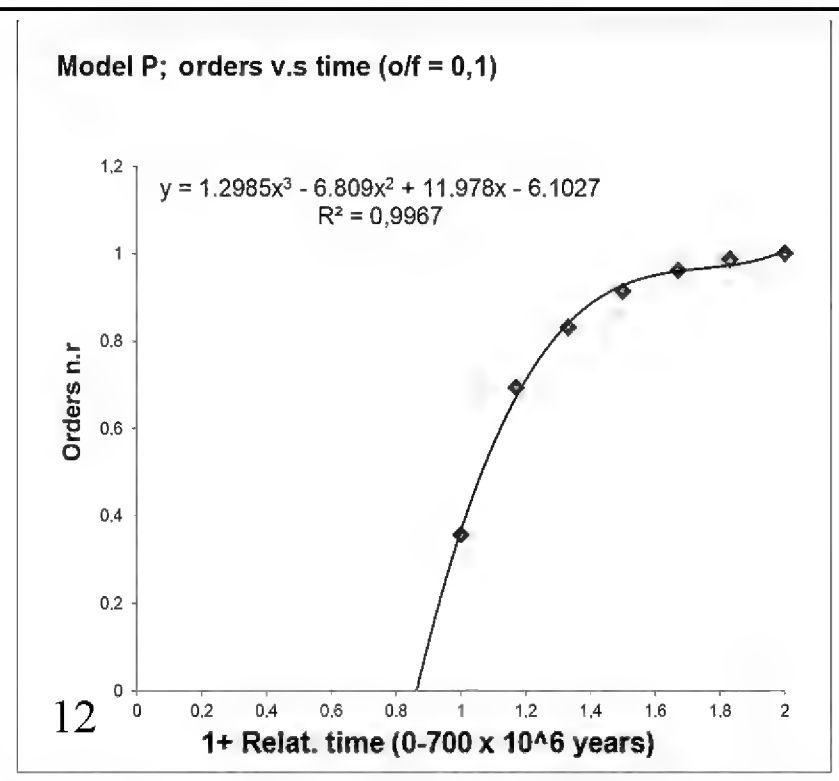
In short, the above approach is mainly devoted to richness component of biodiversity, when the present one concern essentially the numerosity.

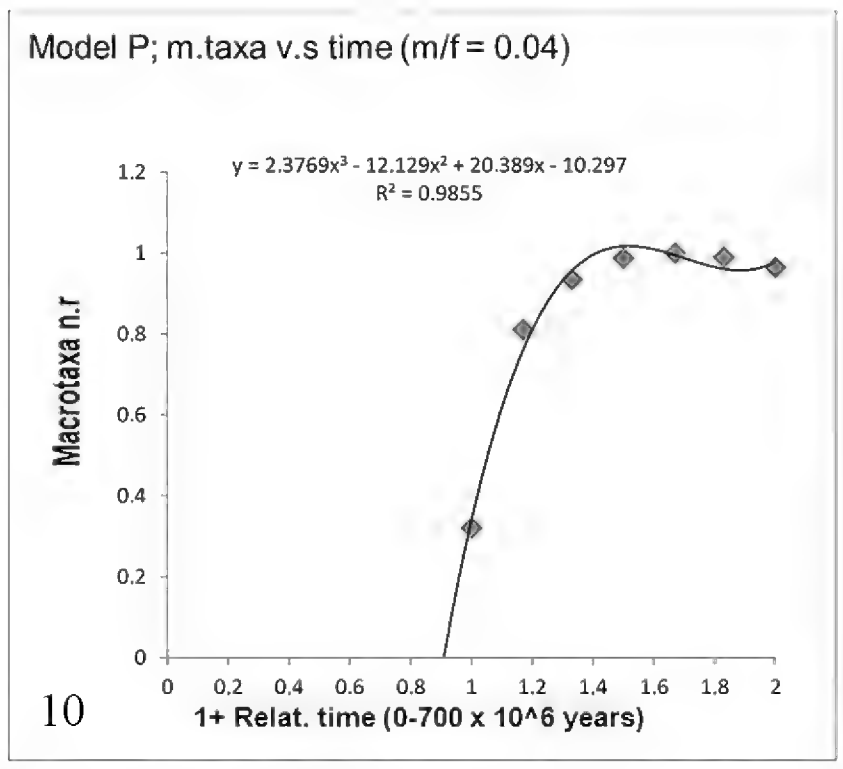
Hence, the two approaches are not antagonist, but are simply linked to different components of biodiversity and different spatio-temporal scales.

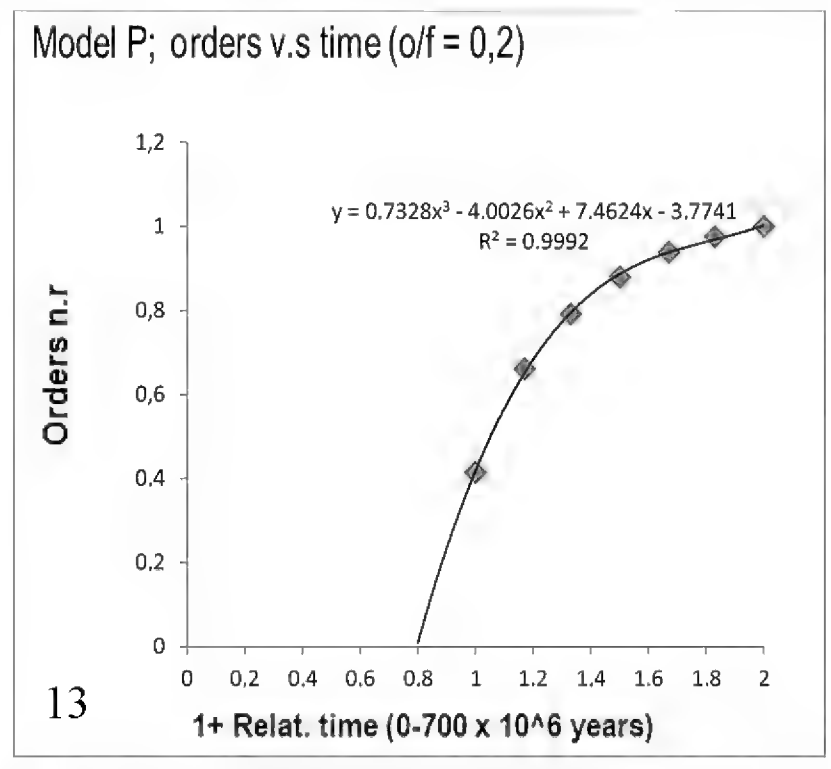
This may partly explain the hyperbolic trend found by Markov & Korotayev (2007) and Dmitriev (2011). However, the general pattern and the determinism of such aproaches do not contrast with the present analysis. Thus, given the above-mentioned approaches, so different in the estimation of changes in palaeontological biodiversity, it was not considered to be appropriate to modify, in a hyperbolic key, the exponential trend deduced, e.g., by Sepkoski (1967, 1979, 2002) and (Benton, 2001).

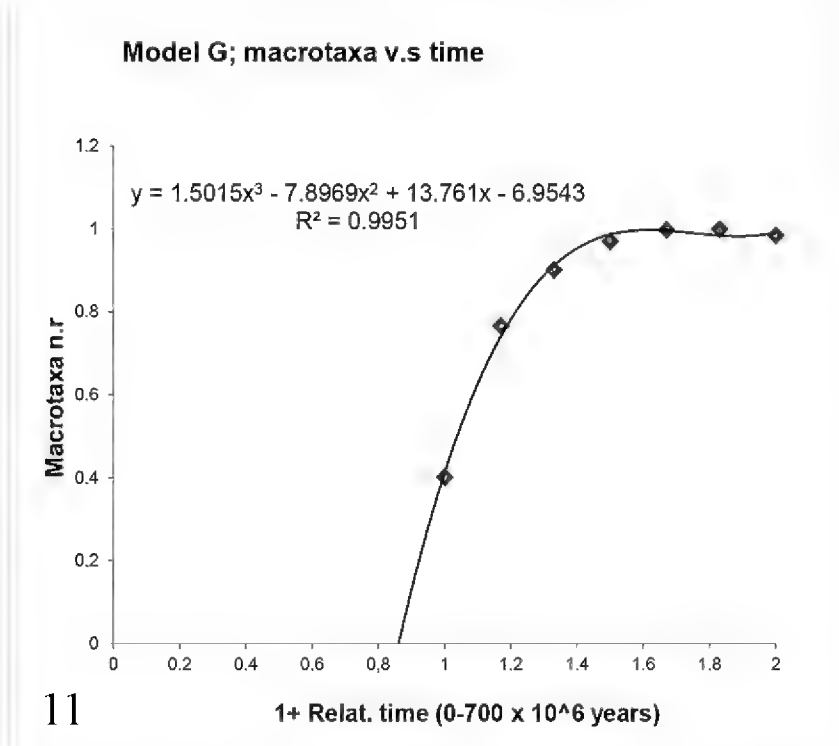
Notwithstanding the great methodological differences, the results of the two approaches on the taxonomical diversity in the Phanerozoic (see Sepkoski, 1967, 1979, 2002; Benton, 2001, followed in this work about numerosity) compared to Alroy, (2008), or Foote (2010), concerning richness, are not irreconcilable (see, e.g., Foote, 2010: fig. 18.6) and do mirror in highlighting two distinct growth phases: a first, apparently self-limiting phase, and a second, nearly autocatalytic phase, with a long "crisis" separation. These results are hence in harmony with the findings of this work, highlighting

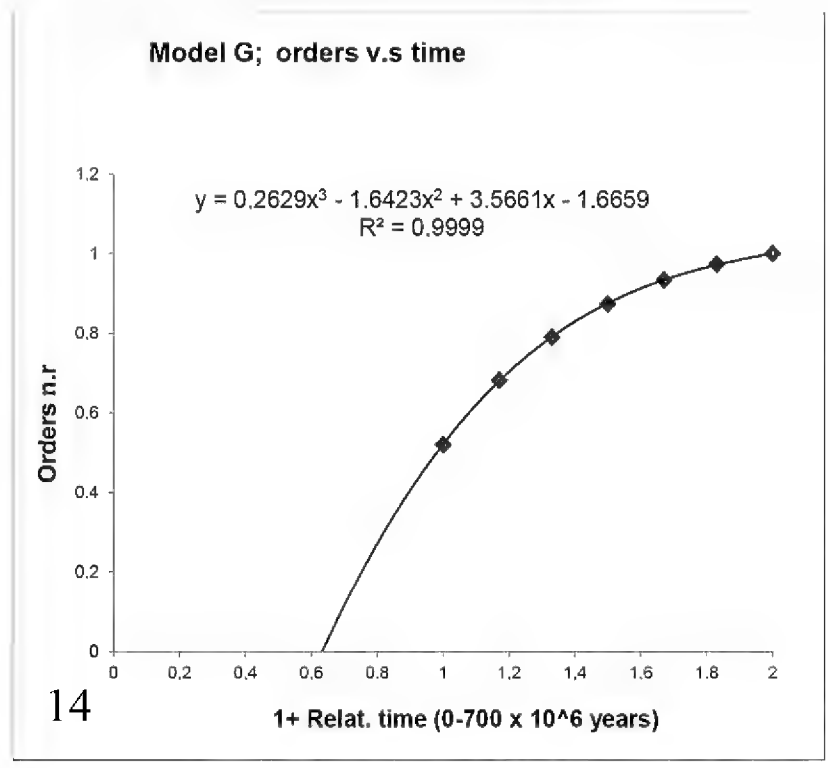












Figures 9–11. Macrotaxa; model P1 (Fig. 9), P2 (palaeontology) (Fig. 10), and G (genetics) (Fig. 11) v.s relative time (0-700 x 10^6 years). P; m/f = hypothesized numerosity ratio "macrotaxa/families". Figures 12–14. Orders ; model P1 (Fig. 12), P2 (palaeontology) (Fig. 13) and G (genetics) (Fig. 14) v.s relative time (0-700 x 10^6 years). P; o/f = hypothesized numerosity ratio "orders/families".

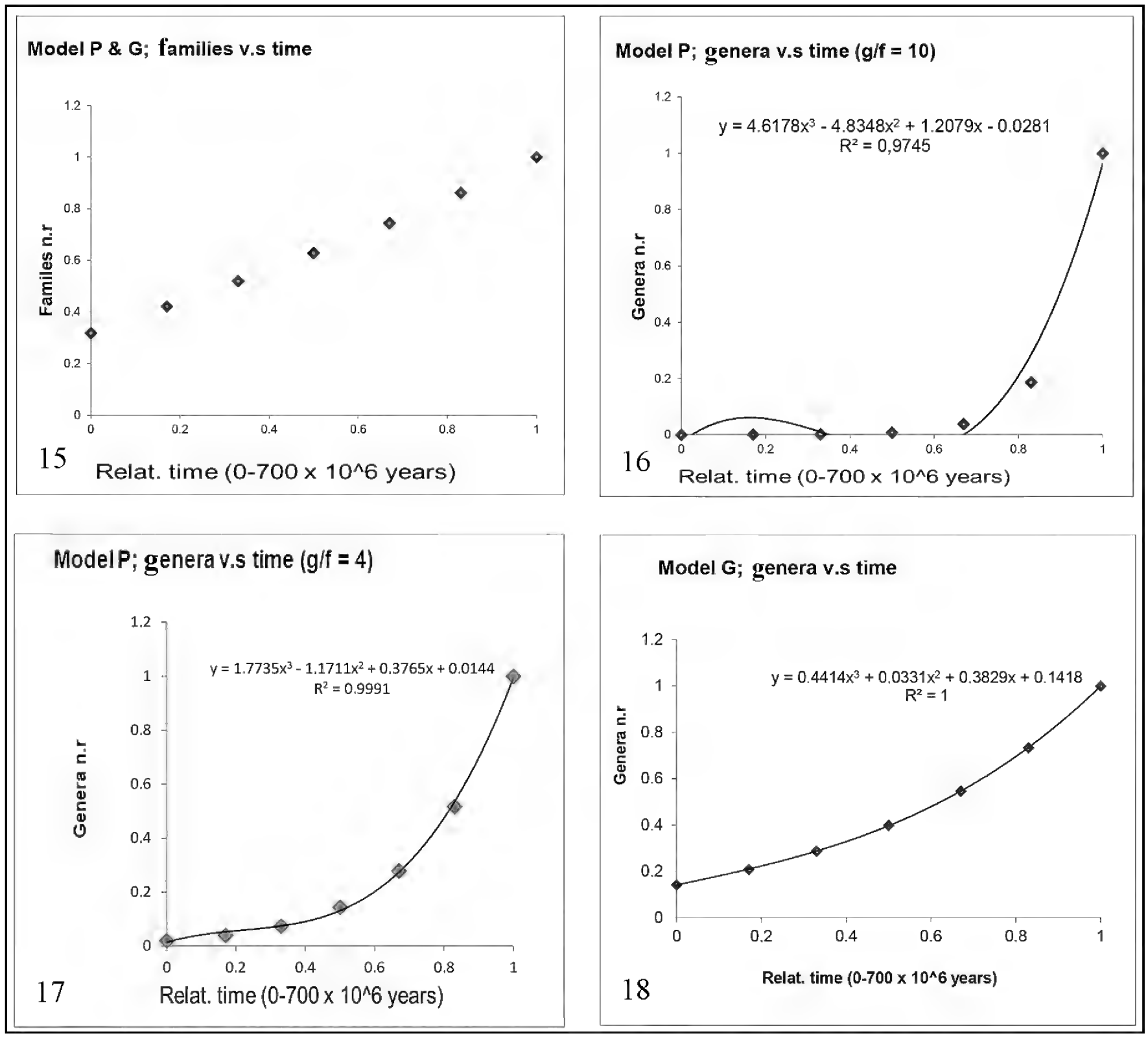


Figure 15. Families; model P (palaeontology) and G (genetics) v.s relative time (0-700 x 10^6 years). Figures 16–18. Genera; model P1 (Fig. 16), P2 (palaeontology) (Fig. 17) and G (genetics) (Fig. 18) v.s relative time (0-700 x 10^6 years). P; g/f = hypothesized numerosity ratio "genera/families".

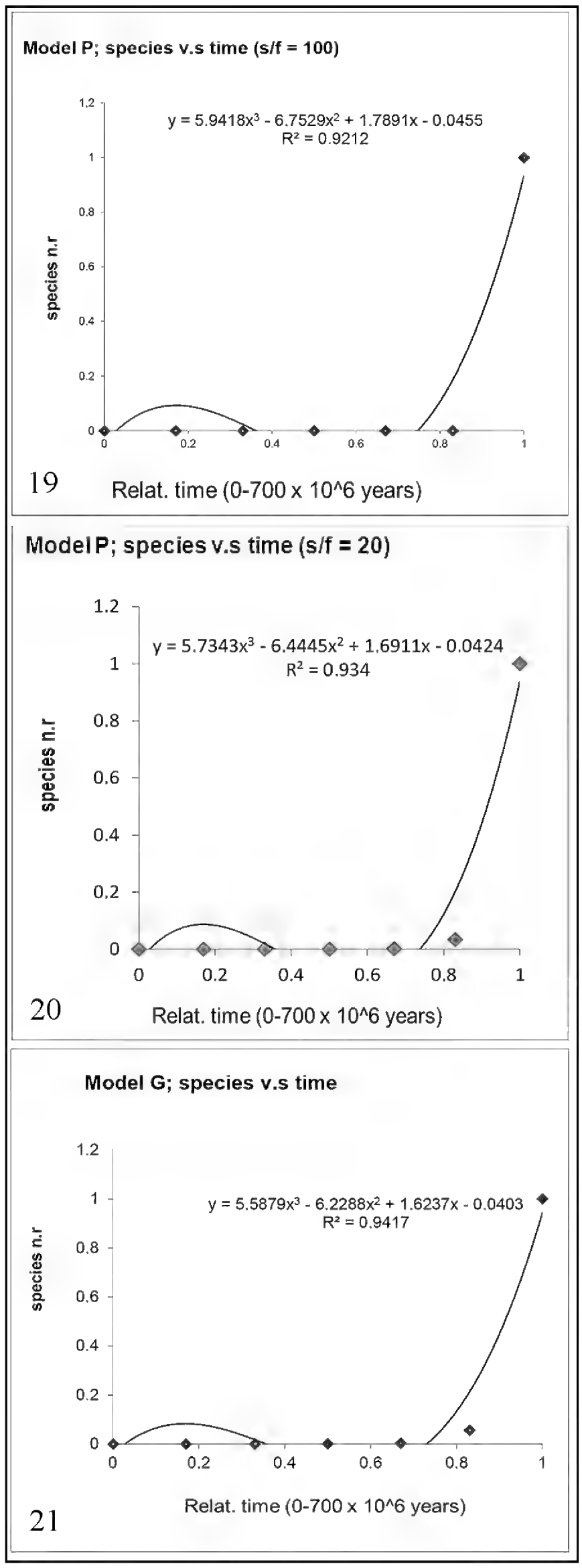
non-obvious differences between macro- and micro-taxa.

Indeed, with time (i) a mainly logarithmic increment followed by a constancy in the number of macro-taxa (i.e., maintenance of phyla and part of the classes with orders being frequently substituted), and (ii) a nearly exponential growth in the number of micro-taxa were observed.

In the above perspective, the intermediate family rank appears to be a quite heterogeneous and perhaps composite one.

Hence, the present results showed an emerging, and not at all merely formal, difference between macro- and micro-taxa as well as between macro- and micro-evolution (Gould, 2002). Indeed, the temporal pattern of the macro-taxa is consistent with a self-limiting model, whereas that for the micro-taxa is typically self-enhancing.

Whilst the micro-taxa patterns (from species to families) are consistent with expectations of the gradualist theory (evolution is essentially a function of time), since at least the Permo-Triassic crisis, the macro-taxa are remarkably different from such expectations, but in agreement with the assortative hypothesis and not in contrast with Gould (2002) and Eldredge (2015).



Figures 19–21. Species; model P1 (Fig. 19), P2 (palaeontology) (Fig. 20) and G (genetics) (Fig. 21) v.s relative time (0-700 x 10^6 years). P; s/f = hypothesized numerosity ratio "species/families".

To finish, it was observed a clear similarity between the theoretical and the observed patterns in time for macro-taxa (Fig. 2 vs. Fig. 5) contrasting with micro-taxa (Fig. 7 vs. Fig. 3), according to two very different hypotheses.

These results encouraged to suggest a quite simple model which, according to the above hypothesis, can unambiguously explain the biological basis of temporal patterns of relative numerosity of both macro- and micro-taxa, considering also the chosen time scale (10^8 years) which smooths the effect of extinction crisis.

Due to its coarseness, the model shows only schematically the relative numerosity basal trends over time of the various taxonomic ranks, with respect to families.

The scope of the model was not to reflect the fluctuations, perhaps mainly stochastic, linked to the crises of numerosity, particularly during the phases of the "large extinctions", especially evident at family rank; on the other hand, there is a shortcoming in the model: it, by definition, cannot show the variation in the growing rate of the families, considered as stable over time.

Nevertheless, the model seems to be quite robust: the obtained trends show very few variations even if the analysed values of the taxonomic ranks do vary around an o. o. m. (order of magnitude).

The model, when applied to palaeontological or genetical data, shows quite similar results even vs. the observed patterns of the bulk of two main groups of various modellized taxonomic ranks (macrotaxa and orders v.s genera and species) over time.

It seems that the hypothetic prevalence of the assortative or the divisive evolutionary mechanism can be sufficient to explain (even if not at all to prove!) the differing basal trends in time of the larger v.s smaller taxonomic ranks.

Finally in the model, the start of Phanerozoic ("0" time in the analysis and "1+0" in some graphs, for computing purposes) seems to be analogue, for large taxa, to a "veil line", hiding a previous phase of great phyletic diversification.

Is the sampling reliable or is it mere artefact?

An important objection to the palaeontological record analyses is linked to the theoretically predictable pattern of the curves "groups/individuals".

These curves would predict, for the groups, a nearly logarithmic growth up to an upper limit (the real numerosity) positively related to the sample size increases. Indeed, it would be expectable that the sample sizes increase regularly and significantly with time from the geological ages to the present.

This eventual bias would at best explain in part the earliest phase of the macro-taxa curve with time, but certainly not the final phase.

Moreover, the rapid exponential growth of the final phase of the micro-taxa curve (sometime, even towards a kind of saturation of a real ecosystem space; see Machac et al., 2013) is clearly against the above-mentioned predictions, which would be necessarily linked to functions of logarithmic (and not exponential) type.

In general, the high fit of the trends over time with a third degree positive polynomial model seems to be quite different to the logistic one, theoretically expected in such a context.

In addition, it does not seem that the importance and completeness of the palaeontological sampling increased with time in the above-mentioned way (Signor, 1985). On the contrary, there are remarkable oscillations and unpredictable decreases even in recent periods because some taxa are unlikely to fossilize especially under some environmental conditions (Signor, 1985; Gould, 2002).

Thus, the observed patterns cannot be explained by classical "groups/individuals" models.

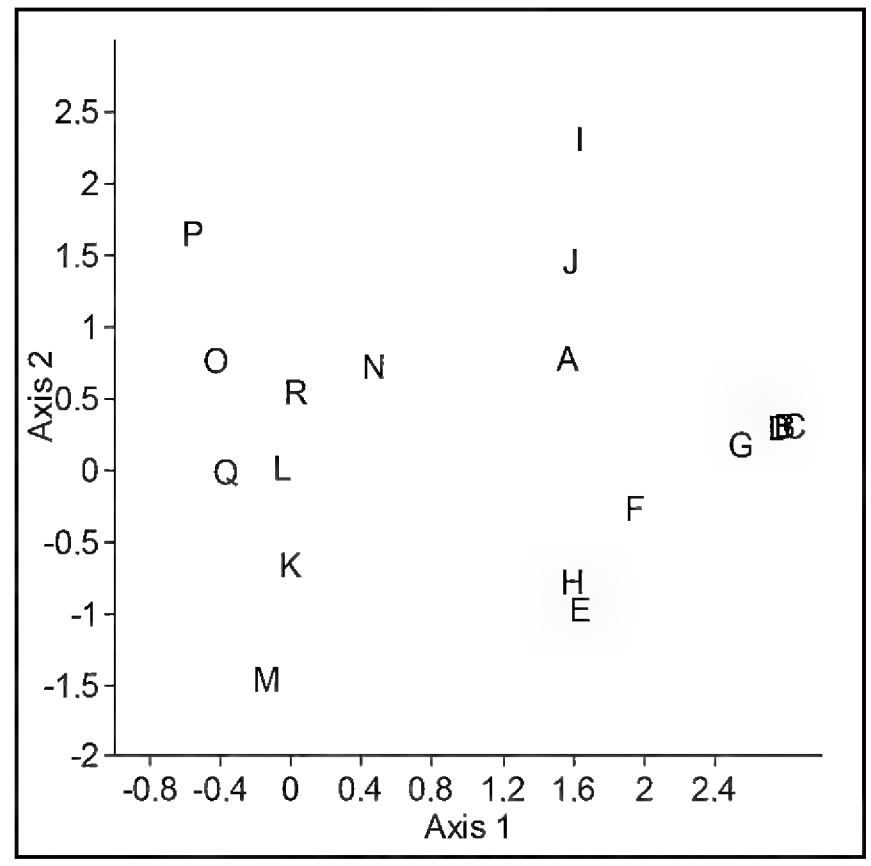


Figure 22. Detrended Correspondence analysis (B, C, and D are overprinted). A: Time; B: species (model P; 20); C: species (model P; 100); D: species (model G;); E: species (observed); F: genera (model P; 4); G: genera (model P; 10); H: genera (model G); I: genera (observed); J: families (observed); K: orders (model P; 0,2); L: orders (model P; 0,1); M: orders (G); N: orders (observed); O: macrotaxa (model P; 0,04); P: macrotaxa (model P; 0,01); Q: macrotaxa (G); R: macrotaxa (observed). Axis 1 = 87 % of total.

Did the observed patterns derive from the evolutive interaction between inter-taxa assortative potentiality and, conversely, subsequent isolation needs for adaptations to reproductive isolation?

Assortative evolution is often considered as exceptionally rare in the present time (however, see Gontier, 2015, without neglecting the hypothesis as brilliant as extreme, between the metaphorical and the paradigmatic, by McInerney et al., 2011) and, possibly, long before the starting of the Phanerozoic (Gould, 2002).

Some doubts about its frequency of occurrence in past times are still not solved, however, some indications have been collected. These can be summarized as follows:

(1) the number of known cases is regularly increasing (Bohm et al., 1997; Chalfie, 1998; Tyler et al., 2006; Gould et al., 2008; Archibald, 2009; Moustafa et al., 2009);

(2) in various taxa, there is still a permanence of karyotypes with high N despite a diffused tendency to Robertsonian fusions (see Wurster & Benirschke, 1967; Morescalchi, 1970; Capanna, 1975);

(3) the occurrence of cases of genetic homologies of structures in very distant clades; these cases are difficult to explain under purely cladogenetic or anagenetic keys of interpretation (Gould, 2002);

(4) the likely prevalence into the genome evolution of an increasing diversification rather than a reductive diversification (Omodeo, 2010).

There have also been some indications of the likely gradual and increasing role played by the inter-taxa isolation over time:

(i) the multiplicity of meiosis models, even within single macro-taxa, suggests that a long time

distance has passed between the emergence of the clades and the fulfilment of each model;

(ii) the great number of inter-taxa reproductive isolation mechanisms, which appeared well after the separation of many large clades;

(iii) the apparently increasing, during the evolution time, of the organic (tegument, digestive - see, e. g., Doolittle, 1998 - immunitary, etc.) barriers against infections, parasitosis, etc.

The general hypothetical scenario: a working hypothesis

Woese (2002, 2004) suggests phases of genetic sharing among organisms, before the splitting of the major evolutionary clades, as Bacteria v.s Archaea and Archaea v.s Eucaria, in connection with some "Darwinian thresholds" (i.e., the time limit after which the "modern" or "Darwinian" species do arise); their increase should be linked to divisive (reproductive isolation, competition etc.) factors.

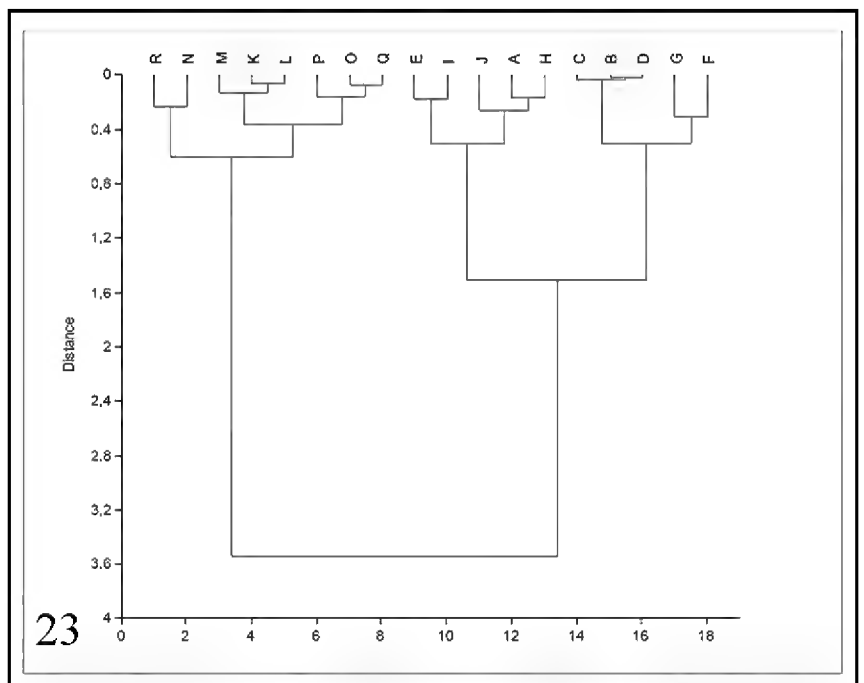


Figure 23. Cluster analysis (Ward). A to R: see above. Figure 24. Recorded Families relative to Max, in time (data from Benton, 2001).

The present analyses support such hypothesis and suggest that the following macroscopic effects of such an assortative phase may justify the trend of larger taxa, up to the early Phanerozoic. According to the model, a kind of "Darwinian threshold" can be connected with the ever growing overcome, in o. o. m. [orders of magnitude], of the species number over that of older clades.

It is likely that the earliest biocoenotic systems were regulated essentially by biochemical and macro-molecular relationships rather than by morphological relationships, as it has surely started to occur in later phases (Gould, 2002; Omodeo, 2010). Hence, based also on the patterns presented in this study (including the sense of "veil line" suggested, in the model, by the Proterozoic-Phanerozoic boundary), it seems possible to confirm that the roots of the Cambrian explosion should be retrodated by far, as the identification of the Cambrian explosion by palaeontological records is inevitably linked to the observation of macro-morphological differences among taxa.

Even genetic data seem in agreement with the above hypothesis: according to figure 2 in Hedges and Kumar (2009), up to about 600 Ma BP estimated genetic divergence did anticipate by hundreds of million of years the palaeontological phenetic one; by this period, both divergences tend gradually to synchronize.

It seems that genetic divergence, in a first (assortative?) phase had preceded and prepared the emerging of more advanced metabionts with complex development (sensu Gould, 2002).

In a second phase, due to the ever growing complexification of adaptations and decreasing probability of an evolutionary advantage of their assortments, possibly the isolation systems among new taxa have become more and more suitable and useful, perhaps also due to a growing "evo-devo" rigidity (Valentine, 1995, 2004; Erwin, 2007).

In this phase, the bulk of genetic divergence could became subsequent to the above phenomenon.

Thus, it is possible that, once overpassed the almost generalized metaclade phase among the early organisms (Penny & Poole, 1999; Gogarten, 2000; Woese, 2004), the potential conditions for the in-

creases in richness of living organisms may have started in a more gradual way and perhaps even before the more evident Phanerozoic explosion.

In the hypothesis of a more ancient and continuous presence of "phanerobionts" in the seas, and in an early phase with environmental niches not yet saturated, there was a prevalence in the living organisms of the assortative evolution innovations, with the origin of nearly all the phyla and of a great part of the classes, in a more biocoenotic than "chemical" context (see Penny & Poole, 1999).

The potentials for the assortative evolution innovations tended to decrease with time in relation to a decreasing probability of qualitatively new combinations.

Later on, in the evolution time, the various adaptations tended to be increasingly refined and specialized, with the risk of altering these delicate and specialized adaptations via assortative evolution became higher than the potential advantages.

Hence, the inter-taxa isolation mechanisms (especially, but not only, the reproductive ones) became prevalent, and the evolution-by-division reached the highest impact on the taxonomic diversification of the biosphere.

Overall, also in light of Woese (2002) results, the present working hypothesis is that there would have been a passage from a phase with global opportunities of structural and metabolic innovations and of their successful genetic combinations (i.e., expressed by the Precambrian and Cambrian explosions) to a phase with prevalent and increasingly rigorous safeguard of the existing adaptations against re-merging which would have been dangerous.

As an expression of a more general pattern consistent with the above-mentioned scenario, there has been also a tendency toward the continuous increment of the relative importance of the DNA control fraction during the evolutionary complexification (Abrahamson et al., 1973; Omodeo 2010).

Obviously, the trend described above was likely accelerated when the global environmental crises (such as the Permian-Triassic one) reduced substantially the numerosity of living taxa, thus creating empty niche spaces for the "new species", which exhibited the stronger and more stable adaptations. It is from these species that the evolution would have re-started, using different modes.

The increasing inter-taxa isolation was also probably enhanced by the new requirements linked to the conquest of terrestrial environment, including, e.g., the internal fecundation requirement, the trophic niche diversification, etc. So, even the new colonization of the seas by taxa returning from the freshwater or the terrestrial environment may have also performed some non-secondary role.

Thus, in conclusion, even towards the end of the Proterozoic, the evolutionary history would have had an early "more cooperative" phase (modulated by inter-taxa innovations through macro-innovation and invasion of new empty niche spaces) and, also, for example, through the Mesozoic "taxa-grinder", a successive phase dominated by competition, with micro-differentiation and safeguard of adaptations and already-conquered niche spaces (modulated by intra-taxa innovations), this latter agreeing with the classical Darwinian theories.

In theory, there should be an obvious, inverse relationship between the taxonomically-based width of the meta-clades and their number on the basis of their respective hierarchic rank.

Also, in the physical sciences, it has been observed that the width of the free spaces covered by useful innovations necessarily decreases with time passing (Kauffman, 1993).

Whereas the present scenario of palaeontological differentiation does not conflict with general physical principles, nonetheless, it does not want to explain the stochastic, oscillating patterns of the different taxa during the different evolutionary times. The results, however, seem to be in part consistent with both the classical "Neo-Darwinism" and with the "Punctuated Equilibria" theories; less, with a strictly gradualist interpretation of the relationships among taxonomic ranks, especially during the early phases of evolution.

In synthesis, the scenario of the work suggests the possibility that the taxonomic biodiversity tends to shift increasingly and inescapably towards least comprehensive and more isolated taxa, i.e. from metaclades to the modern species (Woese, 2002). If confirmed by future results, this process may be hardly reversible, at least for the evolution of metabionts.

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